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Temperature Effects on Growth and Stress Physiology of Brook Trout: Implications for Climate Change Impacts on an Iconic Cold-Water Fish

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Temperature Effects on Growth and Stress Physiology of Brook Trout: Implications for Climate Change Impacts on an Iconic Cold-Water Fish

A Thesis Presented

By

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ABSTRACT

TEMPERATURE EFFECTS ON GROWTH AND STRESS PHYSIOLOGY OF BROOK TROUT: IMPLICATIONS FOR CLIMATE CHANGE IMPACTS ON AN ICONIC COLD-WATER FISH

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Despite the threat of climate change, the physiological mechanisms by which temperature drives the distribution of species are unclear. Here we used chronic temperature exposures to determine that the upper limit for positive growth in the eastern brook trout (*Salvelinus fontinalis*) is 23.4 °C. Additionally, brook trout exposed to daily temperature oscillations of 8 °C, around a mean of 21 °C, exhibited growth rates that were 43 and 35% lower by length and weight respectively, than in constant 21 °C controls. Limitations in growth were associated with increases in indicators of the physiological stress response. Individuals exposed to 22 or 24 °C for 24 days exhibited plasma cortisol levels that were 12 and 18 fold greater than at 16 °C. Similarly, gill heat shock protein (Hsp)-70 levels were 10.7 and 56 fold higher at 22 and 24 °C than at 16 °C. Brook trout exposed to daily temperature oscillation of 4 or 8 °C had gill Hsp-70 levels that were 40 and 700 fold greater than controls. Acute (6 h) temperature exposures were used to demonstrate a threshold for induction of the Hsp-70 and plasma glucose responses of 20.7 °C and 21.2 °C respectively. Finally, we conducted field surveys that demonstrated increased plasma cortisol, plasma glucose, and gill Hsp-70 at temperatures above 21 °C. Induction of the cellular and endocrine stress responses is associated with decreased growth in brook trout. Thermal limitations on growth may provide a mechanism by which temperature drives the distributions of this cold-water species.

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CHAPTER 1

INTRODUCTION

Climate change presents many challenges for the conservation and management of natural resources, yet the mechanisms by which temperature affects populations are often unknown. Although somatic growth is a key aspect of population persistence, our understanding of the means by which temperature impacts growth is limited. Here I assess the physiological responses to climate change for a model species, the eastern brook trout (*Salvelinus fontinalis*). The brook trout is a cold-water species that may be particularly sensitive to climate change, as local populations are spatially constrained to stream networks. My objective is to determine the mechanisms by which thermal stress affects the growth and stress response at the individual.

In spite of its importance to the ecology of cold-water streams and recreational fisheries, the upper limit for growth of juvenile brook trout has yet to be determined. Through recent experiments, I have demonstrated an upper limit for brook trout growth of 23.4 °C. Previous studies indicate the upper lethal limit for brook trout is 25.3 °C (Fry et al., 1946; Fry 1951), yet recent field studies show that brook trout are not present in waters above 22 °C (Wehrly et al., 2007). The greater correspondence of the temperature limits of brook trout in nature to limitations on growth, rather than lethal temperatures, suggests that sub-lethal temperature effects may be most critical to limiting populations.

Our understanding of the mechanisms by which temperature limits growth is incomplete. Elevated temperatures may influence hormonal control of growth through the stress hormone cortisol. Growth Hormone (GH) plays a pivotal role in the endocrine control of growth and energy metabolism in fish (Bjornsson 1997). GH induces production of insulin-like growth factor (IGF)-I, which carries out many of the actions of GH. There is increasing interest in

utilizing mediators of the stress response, such as increased plasma cortisol, glucose, lactate, and Heat shock protein (Hsp) expression as indicators of both individual and population level response to environmental stressors, including temperature (Bonga 1997; Iwama et al., 1999). Our knowledge of thermal effects on the stress response in salmonids is limited and does not extend to brook trout despite a large literature on the species.

The relationship between stress, growth, and the GH/IGF-I axis, is poorly understood in fish (Iwama et al., 1999), though recent results indicate that cortisol may decrease circulating IGF-I (Kajimura et al., 2003b; Peterson and Small 2005a). This relationship is important because of the evidence for sub-lethal temperature limitations on brook trout populations (Wehrly et al., 2007). Sub-lethal temperatures may act via the stress response to inhibit growth in individuals (Portner and Farrell 2008; Wehrly et al., 2007), thus threatening populations. However, there may be inter- or intrapopulation variations in response to increased temperatures, which could provide a resource for evolution in the face of a changing climate (Pulido et al., 2001).

I will examine the relationship between temperature and the stress and growth response in brook trout individuals and populations. **My hypotheses are:**

1) Elevated temperatures will negatively impact growth.

Prediction: Individuals exposed to chronic temperature increases will exhibit decreased growth.

2) Elevated temperatures will induce a stress response.

Prediction: Individuals exposed to acute (hours) and chronic (days) high temperatures will show heightened plasma cortisol, glucose, lactate, and gill Hsp-70 levels.

3) Induction of the stress response by elevated temperature will alter hormonal control of growth.

Prediction: Individuals exposed to chronic high temperatures will exhibit decreased

growth, elevated plasma cortisol levels and altered levels of plasma GH and IGF-I.

Methods: To determine the effects of an acute temperature increase on the stress response, juvenile brook trout will experience a temperature increase (8 °C h^{-1}) from 18 °C to target temperatures of 18, 20, 21, 22, 23, 24, or 26 °C where they will be held for a total of 6 h. Fish will be rapidly anesthetized and blood samples and gill biopsies will be taken. The temperature sensitivity of growth will be assessed by exposing individually marked juveniles to 16, 18, 20, 22, or 24 °C. Length and mass will be assessed for each fish every 8 days until the trial completion at 24 days. Eight fish per group will be sampled as described every 8 days until the trial completion. In order to establish the influence of multiple acute temperature increases on stress and growth, individually tagged fish will be exposed to daily temperature oscillations of 4 or 8 °C around a mean of 21 °C and compared to controls held constant at 21 °C. Length and mass will be assessed as described and tissues samples will be collected as described. Finally, to determine if we can detect bioindicators of temperature stress in wild fish, nonlethal blood and gill biopsies will be collected from wild brook trout at 8 sites in the Connecticut River valley of western Massachusetts in July and November.

Plasma glucose and lactate will be measured using enzymatic coupling assays (Carey and McCormick 1998). Plasma cortisol will be measured using an enzyme immunoassay (Carey and McCormick 1998). Plasma GH and IGF-I levels will be determined using homologous radioimmunoassay (Bjornsson et al., 1994; Moriyama et al., 1994). Salmonids are one of the few fish species for which validated immunoassays for these hormones are available. Hsp-70 levels will be measured through the use of western blotting with a homologous antibody specific to the inducible form of salmonid Hsp-70 (Pelis and McCormick 2001; Rendell et al., 2006). All of these assays are standard in the McCormick laboratory.

By determining the relationships between temperature, stress, and growth in brook trout, this project will provide evidence for temperature effects on growth as a critical mediator of ecological responses to climate change (Portner and Farrell 2008). This model will address an important gap in our understanding of the impacts of climate change on the physiology of wildlife, thus informing climate modelers, ecologists, and wildlife managers. Temperature induced biomarkers will be developed and validated for use in monitoring and assessing the impacts of climate change on fish and wildlife populations.

CHAPTER 2

UPPER THERMAL LIMITS OF GROWTH IN BROOK TROUT AND THEIR RELATION TO STRESS PHYSIOLOGY

Summary

Despite the threat of climate change, the physiological mechanisms that drive important aspects of performance at high temperatures remain unclear for most species. Elevated, but sublethal temperatures may act via mediators of the stress response to limit performance in important life history traits such as growth. Here brook trout subjected to chronically elevated temperatures for 8 and 24 d were monitored for growth and physiological stress responses. Growth rate ($g \bullet d^{-1}$) decreased at temperatures above 16 °C and was negative at 24 °C, with an estimated upper limit for positive growth of 23.4 °C. Plasma cortisol increased with temperature and was 12 and 18 fold higher at 22 and 24 °C than at 16 °C; however, we observed no relationship between temperature and plasma glucose. Gill heat shock protein-70 abundance increased with temperature and was 10.7 and 56 fold higher at 22 and 24 °C than at 16 °C. There was no relationship between temperature and plasma Cl^-, but there was a 53 and 80% decrease in gill Na^+/K^+-ATPase activity and abundance at 24 °C in comparison to 16 °C. Our results demonstrate that elevated temperatures induce cellular and endocrine stress responses and provide a possible mechanism by which growth is limited at elevated temperatures. Temperature limitations on growth may play a role in driving brook trout distributions in the wild.

Introduction

Climate change is the largest ecological challenge that conservationists face today, and will provide many obstacles to those attempting to manage natural resources. In spite of the increasing attention to the ecological impacts of climate change, it is still unclear what physiological mechanisms will operate to drive the responses of individual species to climate change (Helmuth 2009; Portner et al., 2009; Portner and Farrell 2008; Wikelski and Cooke 2006). Although somatic growth is a key aspect of population persistence, our understanding of the means by which temperature impacts growth is limited. Sub-lethal temperatures may act via the stress response to inhibit growth in individuals, thus constraining populations.

The eastern brook trout (*Salvelinus fontinalis*) is an iconic cold-water species of North America and for many stream systems the most abundant vertebrate. Brook trout may be particularly sensitive to climate change, as local populations are spatially constrained to stream networks. Recent brook trout habitat models suggest climate change will lead to a significant loss of habitat throughout the species range, with increasing impacts felt by southern populations (Flebbe et al., 2006; Meisner 1990). These models currently rely on untested assumptions of the upper temperature limit of persistence and would be improved with information on the upper thermal limits for growth in this species. There are well defined effects of temperature on growth rates for most fresh water salmonids (Brett 1979a), but the upper limit for growth of juvenile brook trout has yet to be determined. A number of studies have found 13-16 °C to be optimal for brook trout growth (Baldwin 1956; Dwyer et al., 1983; Hokanson et al., 1973; McCormick et al., 1972; McMahon et al., 2007b), yet none of these studies tested temperatures high enough to determine an upper threshold for growth. Recent field studies indicate that brook trout are not present in waters above a 24 day mean maximum temperature of 22 °C (Wehrly et al., 2007). Additional work in a lentic system suggests that populations are limited by temperatures above 20 °C (Robinson et al., 2010). Laboratory and

field based studies suggest the upper incipient lethal temperature for brook trout is 25.3 °C (Fry et al., 1946; Fry 1951; Wehrly et al., 2007). The disparity between the lethal temperature and the temperature limits seen in nature suggest that sub-lethal temperature effects play a critical role in limiting brook trout populations. Temperature limitations on growth may be one mechanism by which sub-lethal temperatures act to limit brook trout populations.

There is increasing interest in utilizing mediators of the stress response, such as increased plasma cortisol, glucose, lactate, and heat shock protein expression as indicators of both individual and population level response to environmental stressors (Iwama et al., 1999; Iwama et al., 2004; Lund et al., 2002; Wikelski and Cooke 2006). One such stressor is temperature (Wendelaar Bonga 1997); however, our knowledge of thermal effects on the stress response in salmonids is somewhat limited. Experimental warming of juvenile Chinook salmon and rainbow trout resulted in elevated circulating cortisol and glucose levels (Meka and McCormick 2005; Quigley and Hinch 2006). Similarly, adult sockeye salmon exhibited elevated plasma cortisol and lactate levels in response to increased temperature and exercise (Steinhausen et al., 2008). A number of laboratory studies have identified elevated Hsp expression in response to temperature increases in a variety of salmonid species (Dubeau et al., 1998; Lund et al., 2003; Mesa et al., 2002; Rendell et al., 2006; Smith et al., 1999), yet the efficacy of the Hsp response as a biomarker for thermal stress in salmonids has not been fully explored. To date there is limited published information on thermal effects on brook trout stress physiology despite a large literature on the species. Sub-lethal temperatures may act via the stress response to inhibit growth in individuals (Iwama et al., 1999), potentially limiting populations.

In order to better understand the relationship between temperature, stress, and growth in brook trout we exposed fish to constant elevated temperatures (16, 18, 20, 22, or 24 °C) for 8

or 24 days. In addition to growth, we also collected blood and gill tissues in order to assess biomarkers for stress, including gill Hsp-70 and plasma glucose and cortisol. Additionally, we examined gill Na^+/K^+-ATPase activity and abundance and plasma Cl^- to explore the relationship between temperature and osmoregulation.

Materials and methods

Fish stock

Juvenile (0+) brook trout were obtained from the Sandwich State Hatchery (Sandwich, MA, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in July 2009. Fished were housed in 1.7 m diameter tanks supplied with 4l min^{-1} chilled Connecticut river water (16±2 °C) and given supplemental aeration. Fish were fed to satiation (Zeigler Bros, Gardners, PA, USA) with automatic feeders and maintained under natural photoperiod.

Temperature treatment

One week before the start of the experiment eighty fish were removed from their rearing tank and lightly anesthetized with tricaine methanesulfonate (50 mg MS-222 l^{-1}, pH 7.0). While anesthetized, the fish were measured for length (nearest 0.1 cm) and weight (nearest 0.1 g) and implanted with a passive integrated transponder (PIT) tag. PIT tags were inserted into the abdominal cavity via a small (~5 mm) incision in the ventral surface just rostral to the pelvic fins. After recovering from the anesthetic the fish were divided randomly into five 0.9 m diameter experimental tanks (n = 16 per tank). The tanks were supplied with Turners Falls, MA, USA city water at a rate of 0.9 l min^{-1} and held at 16 °C with bayonet heaters (Jalli, 800 Watt Titanium). Each tank was provided with supplemental aeration. Fish were fed to satiation twice daily

throughout the experiment and the amount of feed offered was measured by weight (nearest 0.1 g).

Feed was withheld from the fish for 24 h prior to the start of the experiment and at any other time that length and weight were measured. Seven days after PIT tags were implanted, fish were again measured for length and weight and returned to their appropriate tank. Water temperature was then elevated at a rate of 2 °C h^{-1} until the target temperatures of 16, 18, 20, 22, or 24 °C were reached. Each tank was held at its specific target temperature for the remainder of the study. The water temperature of each tank was measured and recorded every 15 min using Hobo pendent temperature loggers (Onset Computer Corporation, Bourne, MA, USA). Water flow rate and feeding regime were as described with a 75% water change every 4 days. Dissolved oxygen levels were measured daily and were always above 90% saturation.

The fish were measured for length and weight every 8 d for 24 d. On the eighth day half of the fish (n = 8 per tank) were sacrificed using a lethal dose of anesthetic (100 mg MS-222 l^{-1}, pH 7.0) so that blood and tissue samples could be taken. The remainder of the fish (n=8 per tank) were sampled after 24 d. Blood was collected from the caudal vessels using 1 ml ammonium heperanized syringes within five minutes of tank disturbance. The blood was spun at 3200 g for 5 min at 4 °C and the plasma was aliquoted and stored at -80 °C. A biopsy of four to six gill filaments was taken from the first arch and immersed in 100 µl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and stored at -80 °C. The liver was removed, weighed (nearest 0.0001 g), and stored at -80 °C.

Hematocrit

Blood for hematocrit measurement was collected in heperanized micro-hematocrit capillary tubes and centrifuged at 13,500 g for 5 minutes in a micro-hematocrit centrifuge and

read on a micro-capillary reader (Damon/IEC Division, Needham, MA, USA). Here hematocrit is expressed as the percentage of the total blood volume made up of red blood cells.

Gill Na^+/K^+-ATPase activity

Na^+/K^+-ATPase (NKA) activity in gill homogenates was determined using a temperature regulated microplate method (McCormick 1993). Gill biopsies were homogenized in 150 µl SEID (SEI buffer and 0.1% deoxycholic acid). Ouabain-sensitive ATPase activity was measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase in the presence and absence of 0.5 mM ouabain. Samples (10 µl) were run in duplicate in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA). Protein concentration of the homogenate was determined using a BCA protein assay.

Western Blot

Gill heat shock protein-70 (Hsp-70) and NKA protein abundance was measured as previously described (Pelis and McCormick 2001). The remaining homogenate from the gill NKA activity assay was diluted with an equal volume of 2x Laemmli buffer, heated for 15 minutes at 60 °C and stored at -80 °C. Thawed samples were run on a 7.5% SDS-PAGE gel at 2.5 µg per lane with 5 µg Precision Plus protein standards in a reference lane (Bio-Rad Laboratories, Hercules, CA, USA). For each gel two additional reference samples were run, one for Hsp-70 and one for NKA analysis. Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membranes (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mM Tris, 192 mM glycine buffer at pH 8.3. PVDF membranes were blocked in phosphate-buffered saline with 0.05% Triton X-100 (PBST) and 5% non-fat dry milk for 1 h at room temperature, rinsed in PBST, and probed with an Hsp-70 antibody (AS05061; Agrisera, Sweden) diluted 1:20,000 in PBST and 5% nonfat dry milk for 1 h at room temperature. This antibody is specific to the inducible isoform of

salmonid Hsp-70 and does not recognize the constitutive isoform (Rendell et al., 2006). After rinsing in PBST, blots were exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:10,000 in PBST and 5% nonfat dry milk for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent solution A (ECL A; 396 µM coumaric acid, 2.5 mM luminol, 100 M Tris-Cl pH 8.5) and ECL B (0.018% H2O2, 100 mM Tris-Cl pH 8.5), then exposed to X-ray film (RPI, Mount Prospect, IL, USA). After imaging, blots were rinsed in stripping solution (62.5 mM Tris, 2% SDS, 100mM beta-mercaptoethanol pH 6.7) for 30 minutes at 50 °C to remove antigen. Blots were reblocked and reprobed using an NKA alpha-subunit antibody (alpha5; Iowa Hybridoma Bank) diluted 1:10,000 followed by goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:10,000, following the same protocol. Digital photographs were taken of individual gels and band staining intensity measured using ImageJ (NIH, Bethesda, MD, USA); protein abundance is expressed as a cumulative 8-bit gray scale value. The Hsp-70 and NKA reference lanes on each gel were used to correct for inter-blot differences.

Plasma Analysis

Plasma Cl^- was measured by silver titration using a digital chloridometer (Labconco, Kansas City, MO, USA). Plasma glucose was measured by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase (Carey and McCormick 1998). Plasma cortisol was measured by enzyme immunoassay (EIA) as previously described (Carey and McCormick 1998).

Statistics

All data are presented as mean±standard error. For all analyses the probability of establishing statistical significance was $p \leq 0.05$ and when significant effects were observed the r^2 value was reported. All statistical analyses were performed using Statistica 6.0 (Statsoft, Inc., Tulsa, OK, USA). Daily growth rate in weight was calculated as 100(((natural log of end weight)-

(natural log of start weight))/number of days). Hepatosomatic index was calculated as 100(liver weight/body weight). A general linear model was used to analyze the results of this study. Mean temperature was used as a continuous predictor variable and treatment length served as a categorical predictor and this statistical analysis is presented in the text. For the purpose of simplifying our figures, the 8 and 24 d data were pooled and a polynomial regression using just mean temperature as a predictor variable was used for analysis.

Results

The mean water temperatures varied slightly from the target temperatures and were 15.5, 17.7, 20.0, 22.4, and 24.4 °C respectively over the 24 d (Fig. 2.1). There was one mortality in the 20 °C treatment on day 16 and one in the 24 °C treatment observed on day 23. Through 24 days, specific growth rate was highest at 16 °C (3.2 $g \bullet d^{-1}$) and decreased significantly with temperature to a low at 24 °C (-0.9 $g \bullet d^{-1}$) (Fig. 2.2a). Our regression model suggests that the upper limit for positive growth (0.0 $g \bullet d^{-1}$) for juvenile brook trout is 23.4 °C (Fig. 2.2a). Hepatosomatic index decreased with temperature and was 55% lower at 24 °C than at 16 °C (Fig. 2.2b). There was no effect of treatment length on hepatosomatic index ($r^2 = 0.50$, temperature: $p < 0.01$, treatment length: $p = 0.21$). As temperature increased the amount of feed consumed decreased as did conversion efficiency (Table 2.1).

Plasma cortisol levels were lowest at 16 °C (1.3 $ng \bullet ml^{-1}$) and increased with temperature to a peak of 23.4 $ng \bullet ml^{-1}$ at 24 °C (Fig. 2.3a). There was no effect of length of treatment on plasma cortisol ($r^2 = 0.21$, temperature: $p < 0.01$, treatment length: $p = 0.73$). There was no relationship between temperature and plasma glucose across all treatment lengths (Fig. 2.3b). Abundance of the inducible isoform of Hsp-70 in gill tissue increased with

temperature and was 10.7- and 56.0-fold higher after 24 d at 22 and 24 °C than at 16 °C (Fig. 2.3c,d). There was no effect of treatment length on the gill Hsp-70 response ($r^2 = 0.77$, temperature: $p < 0.01$, treatment length: $p = 0.73$).

Gill NKA activity decreased with temperature and after 24 d was 53% lower at 24 °C than at 16 °C (Fig. 2.4a). Gill NKA activity also decreased with treatment length ($r^2 = 0.45$, temperature: $p < 0.01$, treatment length: $p < 0.01$). Similarly, gill NKA abundance decreased with temperature and was 80% lower at 24 °C than at 16 °C after 24 d (Fig. 2.4b). There was no relationship between treatment length and gill NKA abundance ($r^2 = 0.38$, temperature: $p < 0.01$, treatment length: $p = 0.31$). Hematocrit levels were between 30 and 35% in all treatments except after 24 d at 24 °C where they decreased to a low of 18% (Fig. 2.5a) ($r^2 = 0.32$, temperature: $p < 0.01$, treatment length: $p < 0.01$). There was no relationship between temperature and plasma chloride levels which were similar among all treatments (Fig. 2.5b).

Discussion

Our results indicate a decline in growth rate as temperature increases above 16 °C in brook trout. These results are in line with an extensive literature suggesting optimal growth at 13 °C (Baldwin 1956), 14.3 °C (McMahon et al., 2007a), and 16 °C (Dwyer et al., 1983; Hokanson et al., 1973). None of these studies incorporated enough treatments above the optimal temperature to adequately describe brook trout growth at elevated temperatures. Nor did they test temperatures high enough to determine the upper limit for growth in brook trout. To our knowledge, we are the first to report that the upper limit for positive growth (0.0 $g \bullet d^{-1}$) in brook trout is 23.4 °C. (Wehrly et al., 2007) reported that brook trout are not found in waters above a 24 day mean maximum temperature of 22 °C despite the fact that the lethal temperature in this

species is 25.3 °C (Fry et al., 1946; Fry 1951; Wehrly et al., 2007). The fact that the ecological limit is more closely associated with temperature limitations on growth than it is with the lethal temperature suggests that temperature limitations on growth may play a key role in determining brook trout distributions.

In spite of a well-developed literature in salmonids demonstrating decreased growth at elevated temperatures, the mechanism for temperature control of growth remains unclear. Induction of the stress response by temperature may influence endocrine control of growth. In the present study we observed an increase in plasma cortisol levels as temperature increased above 16 °C, with peak cortisol levels at 22 and 24 °C. Increased cortisol in response to elevated temperatures (Quigley and Hinch 2006), elevated temperature and angling (Meka and McCormick 2005), and elevated temperature and exercise (Steinhausen et al., 2008) has been reported in a number of salmonids and together with our findings, implicate temperature as an endocrine stressor. Cortisol modulates aspects of metabolism such as increasing plasma glucose levels via glyconeogenesis in the liver (Mommsen et al., 1999; Vanderboon et al., 1991; Wendelaar Bonga 1997). In the current study we did not observe a relationship between plasma glucose levels and temperature. However, we have recently observed increased plasma glucose levels in brook trout 6 h after temperatures were increased. (J. G. Chadwick, Jr and S. D. McCormick, unpublished). (Vanlandeghem et al., 2010) observed an increase in plasma glucose in largemouth bass 1 h after heat shock, but not after 6 h. It is possible that glucose responds to acute, but not chronic temperature elevations in brook trout and that we missed the signal due to the timing of our sampling. Increases in cortisol may in fact induce increased glucose production which is matched by increased glucose utilization, resulting in no net increase in plasma glucose. It is also possible that fish at elevated temperatures simply depleted their

hepatic glycogen stores as a result of this chronic thermal stressor. Indeed, we observed a 55% decrease in hepatosomatic index after 24 d at 24 °C compared to 16 °C controls.

In the current study plasma cortisol levels were elevated at temperatures were growth was decreased. Cortisol is an important regulator of metabolism and elevated levels may impact growth through several pathways. Elevated plasma cortisol is known to increase metabolism as it heightens gluconeogenesis in the liver and raises rates of catabolic pathways such as glycolysis and proteolysis (Mommsen et al., 1999; Vanderboon et al., 1991). This increased metabolic rate is important during times of stress as it provides the necessary energy needed by vital organs to maintain homeostasis, but it also diverts resources away from anabolic pathways necessary for growth. We observed a decrease in feed conversion efficiency as temperature increased above 16 °C, suggesting that metabolism was also increased at these temperatures. In the lab, cortisol administration has been shown to increase aerobic and anaerobic metabolism in cutthroat trout and rainbow trout (De Boeck et al., 2001; Morgan and Iwama 1996). More recently, cortisol injection resulted in decreased growth in wild largemouth bass and increased standard metabolic rate in lab reared largemouth bass (O'Connor et al., 2011). Furthermore, exposure to a daily stressor resulted in increased metabolic rate and decreased aerobic scope in green sturgeon (Lankford et al., 2005). In the current study we observed decreased feeding at temperatures that were high enough to induce a cortisol response. This finding is in agreement with a growing literature that demonstrates a negative relationship between cortisol and appetite and feeding rates in fish. In rainbow trout and channel catfish exogenous cortisol administration resulted in decreased feeding and growth rates (Gregory and Wood 1999; Peterson and Small 2005b). Chronic stress and dietary cortisol reduced feed intake and conversion efficiency in sea bass (Leal et al., 2011). Atlantic salmon smolts and rainbow trout exhibited suppressed feeding following an acute confinement stressor (Pankhurst et al., 2008a;

Pankhurst et al., 2008b). The exact mechanism for the suppression of feeding by cortisol is still under investigation though there is evidence that cortisol reduces plasma ghrelin levels (Pankhurst et al., 2008a; Pankhurst et al., 2008b). The suppression of feeding at high temperature may be important for maintaining aerobic scope in fish.

Cortisol, elevated in response to high temperature, may also influence growth through the GH/IGF-I axis. Exogenous cortisol has been shown to reduce IGF-I mRNA and plasma levels in tilapia (Kajimura et al., 2003a). In channel catfish, cortisol administration resulted in reduced growth and plasma IGF-I and increased plasma GH (Peterson and Small 2005b). These findings add to an extensive literature on the effects of starvation in salmonids (Deane and Woo 2009). Nutritional restriction leads to liver GH resistance, a condition in which the down regulation of GH receptor results in decreased plasma IGF-I despite elevated plasma GH (Gray et al., 1992; Perez-Sanchez et al., 1994; Pierce et al., 2005).

In the current study elevated temperature also induced a cellular stress response. Gill Hsp-70 levels increased with temperature and were 10 and 56-fold higher at 22 and 24 °C than they were at 16 °C. There was relatively little gill Hsp-70 at 20 °C, but at 22 °C expression had been induced suggesting a threshold for induction of the Hsp response of between 20 and 22 °C. Acute (hours) exposures in our lab suggest a threshold for induction of between 20.5 and 22 °C in brook trout (J. G. Chadwick, Jr and S. D. McCormick, unpublished). Interestingly, the threshold for the heat shock response is similar to both the upper limits for growth as well as to their upper ecological limit (Wehrly et al., 2007). Acute exposure to elevated temperature has been shown to increase red blood cell Hsp-70 abundance above control levels in brook trout at 25 °C, but not at lower temperatures; however, this study found increased Hsp-70 mRNA at 22 °C in a variety of tissues (Lund et al., 2003). It is likely that the assay used in this study was not as

sensitive as ours due to the use of an antibody that recognizes the constitutive and inducible isoforms of Hsp-70, but there are also likely to be tissue specific differences in Hsp expression patterns. In Atlantic salmon, a close relative with greater thermal tolerances than brook trout, the threshold for induction of Hsp-70 and 30 was found to be between 22 and 25 °C (Lund et al., 2002). Acute exposure to 25 or 26 °C resulted in increased hepatic Hsp-70 levels in rainbow trout and Chinook salmon, but these studies were not designed to determine temperature thresholds for induction (Mesa et al., 2002; Rendell et al., 2006). In addition to these laboratory studies, field based studies have observed a positive relationship between water temperature and Hsp levels in brook trout (J. G. Chadwick, Jr and S. D. McCormick, unpublished) as well as in rainbow trout and Atlantic salmon (Feldhaus et al., 2010; Lund et al., 2002; Werner et al., 2005).

Here we show decreased growth at temperatures high enough to induce gill Hsp-70. The Hsp response is not without cost and may impact growth. It has been suggested that the synthesis of Hsps consumes an inordinate amount of cellular or organismal nutrient stores and could occupy enough of the transcriptional and translational machinery within the cell to hinder other essential biochemical pathways, including those associated with growth (Feder and Hofmann 1999). If this is true then one would expect to see an attenuated Hsp response in individuals subjected to nutritional restriction. In a number of species, starved fish exhibit increased Hsp levels under normal temperature conditions (Cara et al., 2005; Piccinetti et al., 2012; Yengkokpam et al., 2008), but when given a temperature challenge the Hsp response in starved fish is less than that of fed fish (Deng et al., 2009; Han et al., 2012; Piccinetti et al., 2012). Furthermore, a correlation between Hsp induction and reduced metabolic condition has been reported in juvenile steelhead trout (Viant et al., 2003). Currently, the metabolic cost of mounting an Hsp response represents a gap in our understanding of the cellular stress response.

In addition to thermal impacts on brook trout growth and stress physiology we also explored the capacity for osmoregulation at stressfully elevated temperatures. Gill Na^+/K^+-ATPase activity (50%) and abundance (80%) decreased with temperature and were lowest at 24 °C. Despite this there were no differences in plasma chloride levels in our temperature treatments. Elevated temperature has been shown to reduce the length of the smolt window, as characterized by decreased gill Na^+/K^+-ATPase activity and sea water tolerance, in anadromous salmonids (McCormick et al., 1996; McCormick et al., 1999), though this may be more related to temperature impacts on development than it is to temperature effects on osmoregulation. Similarly, elevated temperature also reduces survival and gill Na^+/K^+-ATPase activity in sockeye salmon during their spawning migration (Crossin et al., 2008). An inverse relationship between temperature and gill Na^+/K^+-ATPase activity has been observed in cod (Staurnes et al., 1994), halibut (Jonassen et al., 1999), and pupfish (Stuenkel and Hillyard 1980), but not in turbot (Burel et al., 1996). It is plausible that changes in enzyme kinetics or alterations in behavior at elevated temperatures lessen the demand for both gill Na^+/K^+-ATPase activity and abundance, though more exploration in these areas is clearly needed.

Here we demonstrate reduced growth in juvenile brook trout held at constantly elevated temperatures. These treatments also induced the cellular and endocrine stress responses and potentially reduced the osmoregulatory capacity in these individuals. Growth is an important aspect of life history that affects the reproductive capacity of an individual. In salmonids a clear relationship between body size and fecundity has been demonstrated (Thorpe et al., 1984) and an inverse relationship between temperature and reproduction has been described in wild brook trout (Robinson et al., 2010). Furthermore, reduced body size may increase an individual's vulnerability to predation and decrease its ability to establish territory and exploit food resources. Taken together, the impact of elevated temperature on growth in

brook trout individuals may provide a mechanism by which populations are limited by elevated temperature. We fully acknowledge that the response of any species to a changing environment is dynamic and that changes in growth rate represent one potential response. Elevated temperatures may impact behavior, feeding, and predator avoidance along with a host of other aspects of physiology. Likewise, climatic changes may affect other species with which brook trout interact. Similarly, we also acknowledge that changes in temperature are one aspect of the dynamic process that is climate change. In addition to temperature, there are likely to be other aspects of climate change that will impact brook trout populations.

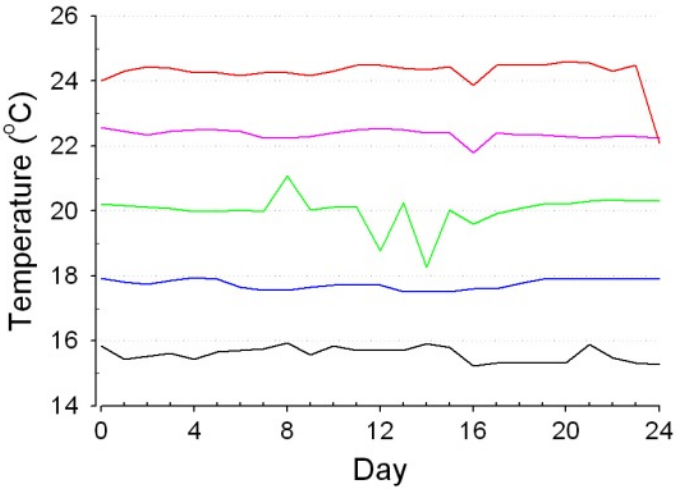

Figure 2.1 | Daily mean water temperature in each of the five temperature treatments.

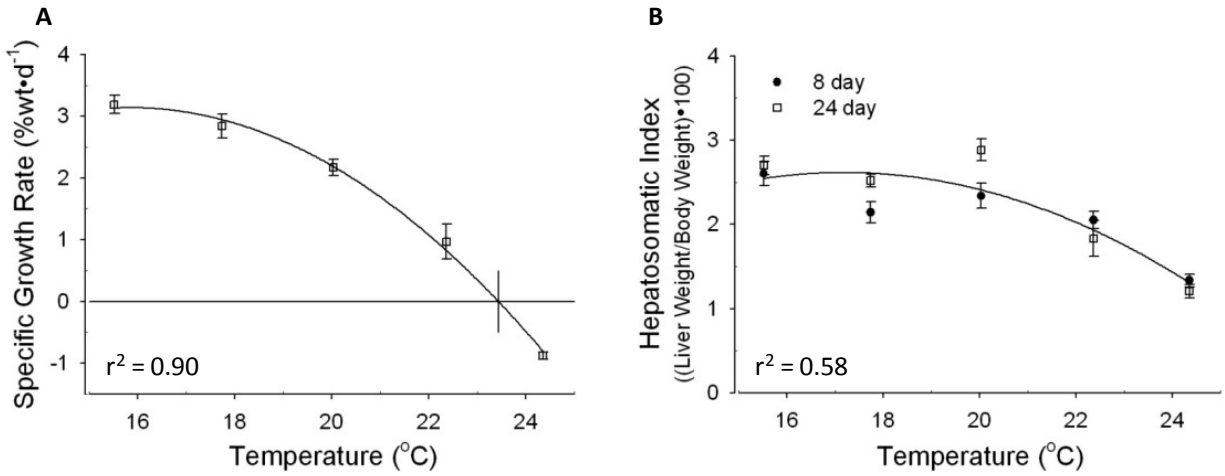

Figure 2.2 | The effect of temperature on (A) growth rate and (B) hepatosomatic index in brook trout. Points represent mean (n=7-8) ± sem. A polynomial regression using mean temperature as the predictor variable was used to analyze the data.

Table 2.1 | The effect of temperature on feeding and feed conversion efficiency. Temperature is the mean water temperature over 24 d. Feed offered is the total dry weight of feed offered per treatment over 24 d. Weight gained was the total dry weight gained per treatment over the 24 d. Conversion efficiency = Weight Gained/Feed Offered.

Temperature (°C)	Feed Offered (g)	Weight Gained (g)	Conversion Efficiency
15.5	86.1	37.2	0.432
17.7	77.4	31.0	0.401
20.0	57.0	19.8	0.347
22.4	45.8	9.7	0.213
24.4	25.0	-9.8	-0.931

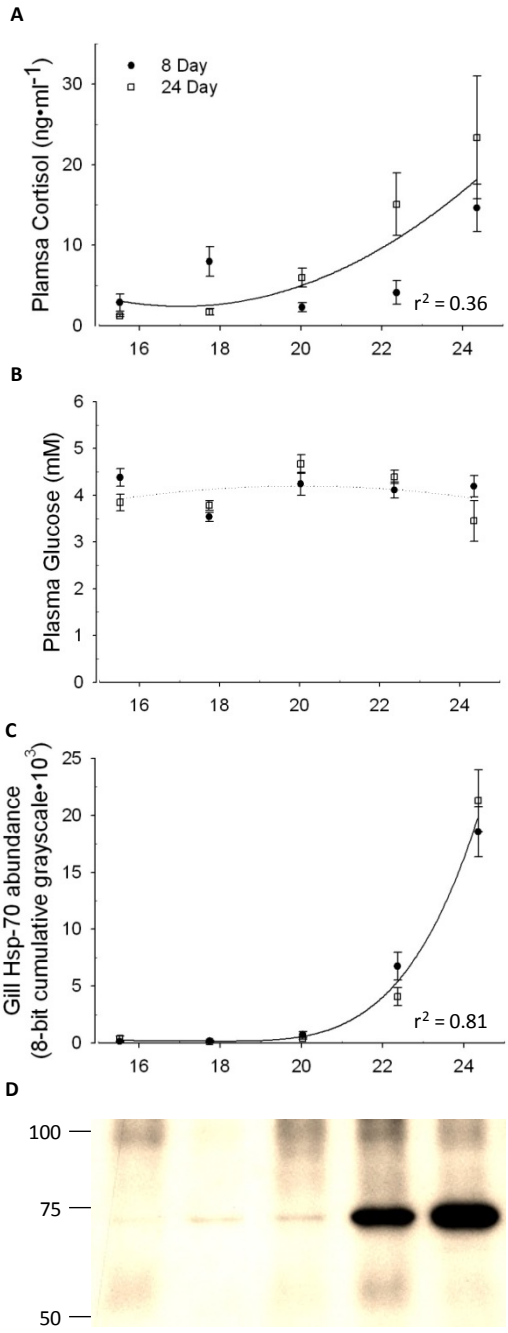

Figure 2.3 | The effect of 8 or 24 d chronic temperature treatment on (A) plasma cortisol, (B) plasma glucose, and (C) gill Hsp-70 abundance in brook trout. Points represent mean (n=7-8) ± sem. A polynomial regression using mean temperature as the predictor variable was used to analyze the data. (D) A representative western blot of Hsp-70 immunoreactivity in gill tissue. Size markers are in kDa.

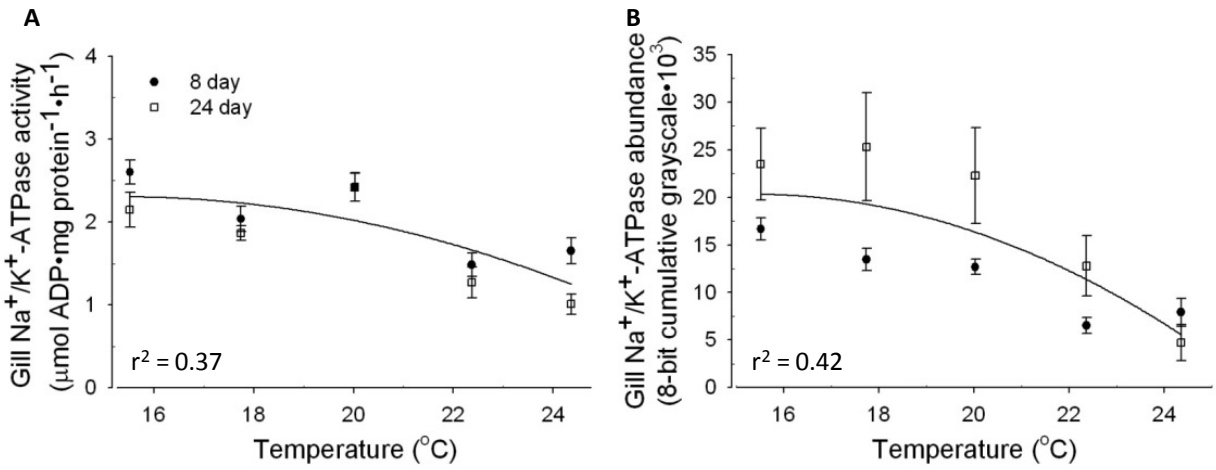

Figure 2.4 | The effect of 8 or 24 d chronic temperature treatment on (A) NKA activity and (B) abundance in brook trout. Points represent mean (n=7-8) ± sem. A polynomial regression using mean temperature as the predictor variable was used to analyze the data.

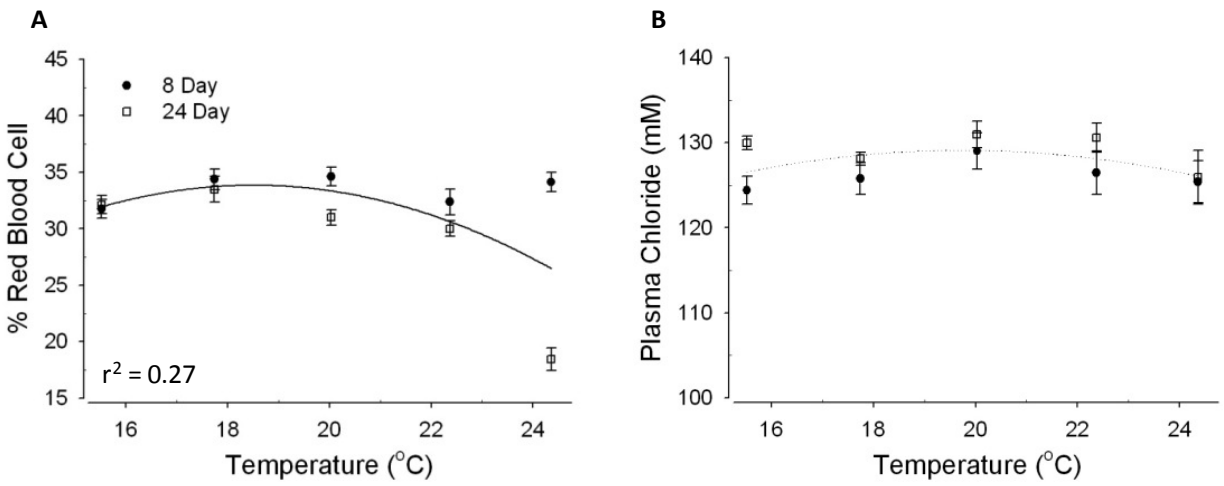

Figure 2.5 | The effect of 8 or 24 d chronic temperature treatment on (A) hematocrit and (B) plasma chloride in brook trout. Points represent mean (n=7-8) ± sem. A polynomial regression using mean temperature as the predictor variable was used to analyze the data.

CHAPTER 3

EFFECT OF DAILY TEMPERATURE OSCILLATION ON GROWTH AND STRESS PHYSIOLOGY IN BROOK TROUT

Abstract

We investigated the effect of daily temperature oscillations on growth and stress in brook trout (*Salvelinus fontinalis*). Individuals acclimated to 21 °C were exposed to daily temperature oscillations of 4 or 8 °C (19-23 °C or 17-25 °C) and compared to 21 °C controls. Growth rate decreased with temperature and was 43% and 35% lower by length and weight in the 8 °C daily oscillation treatment than in the controls. There was no effect of temperature oscillation on plasma cortisol or glucose levels. In contrast, gill heat shock protein (Hsp)-70 abundance increased with increasing daily oscillation. In individuals that were exposed to these daily oscillations for 8 or 24 d gill Hsp-70 abundance was 40 and 700 fold greater at 4 and 8 °C daily oscillation than in the constant temperature controls. In individuals exposed to 8 °C diel oscillations for 4 d and then allowed to recover at 21 °C, gill Hsp-70 abundance was still elevated after 4 d recovery, but not after 10 d. Induction of the cellular stress response by oscillating temperature is associated with reduced growth and indicates that daily temperature oscillations may have ecological effects and distributional impacts on cold-water species.

Introduction

Climate change is likely to have profound and long lasting effects on ecological systems. There has been an increasing focus in the scientific literature on the impacts of elevated temperature on various aspects of physiology and whole animal performance (Helmuth 2009; Portner and Farrell 2008; Somero 2012; Wikelski and Cooke 2006). In most cases individuals are exposed to acute (hours) or chronically (days and weeks) elevated fixed temperatures. However,

these constant exposure experiments are inconsistent with what most species experience in the wild. Daily variations in temperature are a normal part of life in most habitats. As the climate warms many species will experience daily temperature oscillations that feature stressfully elevated temperatures. Here we investigate the effects of such daily temperature oscillations on an iconic cold-water fish species, the eastern brook trout (*Salvelinus fontinalis*). Brook trout may be particularly sensitive to the effects of climate change as populations are spatially constrained to stream networks that contain the cold clean water that they depend upon. Indeed, recent habitat models suggest that brook trout will experience a decline in their range as a result of climate change, with such effects being disproportionately felt in the southern end of their distribution (Eaton and Scheller 1996; Flebbe et al., 2006; Meisner 1990). These models are based upon the relationship between temperature and their current distribution and incorporate future climate predictions; however, uncertainty regarding the relationship between elevated temperature, the stress response, and overall performance in individuals reduces the confidence in such models, which currently do not incorporate the impacts of daily variations in temperature.

The relationship between growth and temperature has been well described for a variety of salmonid species (Baldwin 1956; Brett 1979b; Dwyer et al., 1983; McCormick et al., 1972; McMahon et al., 2007a); however, growth in brook trout at elevated temperatures has received relatively little attention. Recent work from our lab using chronic temperature exposures demonstrated reduced growth in juvenile brook trout as temperature increased above 16 °C (Chadwick and McCormick, unpublished data). In this study, the upper limit for growth in brook trout was determined to be 23.4 °C. Extensive field sampling in lotic habitats suggests that brook trout are rarely observed in the wild above a 24 day mean temperature of 22 °C (Wehrly et al., 2007) whereas in lentic habitats they have been shown to be limited at temperatures above 20

°C (Robinson et al., 2010). This is in contrast to lab and field based studies that demonstrate a lethal limit of 25.3 °C in this species (Fry et al., 1946; Fry 1951; Wehrly et al., 2007). The greater proximity of the ecological limit to thermal limitations on growth than to the lethal limit suggests that temperature limitations on growth play an important role in regulating brook trout distributions. However, relatively little attention has been paid to the impact of daily temperature oscillations on growth in salmonids. At elevated temperatures, rainbow trout exhibited decreased growth in fluctuating treatments compared to constant controls (Hokanson et al., 1977). Growth rate in Lahontan cutthroat trout decreased with increasing magnitude of daily oscillation in comparison to a constant temperature control (Meeuwig et al., 2004). In Atlantic and coho salmon, exposure to daily temperature oscillations did not alter growth, but these studies were conducted at temperatures that were near optimal for growth (Shrimpton et al., 2007; Thomas et al., 1986).

Stress is an important response to high temperature and may influence growth and survival. It has been thoroughly demonstrated that cortisol is the stress hormone in fish as it is in other vertebrates (Bondy et al., 1957; Mommsen et al., 1999; Wedemeyer 1969; Wendelaar Bonga 1997). In fish, the hypothalamic-pituitary-interrenal axis is activated and releases cortisol in response to both real and perceived stressors (Ilan and Yaron 1980; Mommsen et al., 1999; Sumpter et al., 1986; Wendelaar Bonga 1997). A primary function of cortisol in the stress response is the adjustment of various metabolic pathways, including mobilizing energy stores via stimulation of gluconeogenesis in the liver (Mommsen et al., 1999; Vanderboon et al., 1991; Wendelaar Bonga 1997). The relationship between elevated temperature and the endocrine stress response has not been well characterized in most salmonids. Chinook salmon and rainbow trout sampled at high temperature exhibited increased plasma cortisol and glucose levels, while increased temperature and exercise resulted in elevated plasma cortisol and lactate

in adult sockeye salmon (Meka and McCormick 2005; Quigley and Hinch 2006; Steinhausen et al., 2008). Most studies that have examined daily temperature fluctuations on salmonid growth either did not measure plasma cortisol or did not test temperatures high enough to see a relationship with temperature (Hokanson et al., 1977; Meeuwig et al., 2004; Shrimpton et al., 2007). However, in coho salmon plasma cortisol was significantly higher in fish exposed to a 6.5 – 20 °C daily fluctuation than in individuals exposed to a more moderate fluctuation or to constant temperatures (Thomas et al., 1986).

In addition to the endocrine stress response, induction of the cellular stress response by temperature may also influence growth and survival. A growing literature has demonstrated an increase in Hsp expression in fish in response to a number of environmental stressors, including elevated temperature (Deane and Woo 2011). Inducible isoforms of Hsps are upregulated in order to protect the cell from the potentially toxic presence of denatured proteins by binding to denatured proteins and where possible refolding them into their native configuration. When protein rescue is not possible, Hsps play a role in sequestering and recycling these denatured proteins, thus preventing the formation of potentially toxic protein aggregates (Deane and Woo 2011; Tomanek 2010). A number of studies have documented the induction of Hsp-70 expression in response to temperature stress in a variety of salmonid species (Dubeau et al., 1998; Mesa et al., 2002; Rendell et al., 2006; Smith et al., 1999), including brook trout (Lund et al., 2003).

Despite an emerging literature linking elevated temperature to decreased growth, it remains unclear what mechanisms are acting to drive this trend. The endocrine and cellular stress response may act through several pathways to reduce growth at elevated temperatures. The endocrine stress response may influence hormonal control of growth. Exogenous cortisol has been shown to reduce growth and plasma insulin-like growth factor (IGF)-I in fish (Kajimura

et al., 2003b; Peterson and Small 2005a). Elevated cortisol has also been demonstrated to increase metabolic rate (De Boeck et al., 2001; Lankford et al., 2005; Morgan and Iwama 1996; O'Connor et al., 2011) and reduce appetite in fish (Davis and Peterson 2006; Gregory and Wood 1999; Leal et al., 2011; Pankhurst et al., 2008a; Pankhurst et al., 2008b). The cellular stress response is also likely to be costly as it consumes resources and occupies the transcriptional and translational machinery of the cell (Feder and Hofmann 1999).

There is extensive and increasing attention being paid to the use of mediators of the stress response, such as heat shock protein (Hsp) expression and plasma cortisol and glucose, as biomarkers for environmental stress (Iwama et al., 1999; Iwama et al., 2004; Lund et al., 2002; Wendelaar Bonga 1997; Wikelski and Cooke 2006). Despite this attention, the efficacy of Hsp-70 as a biomarker for temperature stress in wild populations has not been adequately explored. The threshold for induction of Hsp-70 has not been established in most species nor has the duration of the signal once the stressor is removed. Furthermore, it is unknown how daily exposure to an acute thermal stress will impact the endocrine and cellular stress response. This relationship is important as wild populations are likely to experience brief, but stressful temperatures more frequently in warming climate.

We contend that a daily temperature oscillation that features a peak temperature above 21 °C will result in reduced growth and induction of the endocrine and cellular stress responses in brook trout. In order to investigate the effects of daily temperature variations on growth and stress in brook trout, individuals acclimated to 21 °C were exposed to daily temperature oscillations of 4 or 8 °C (19 to 23 °C or 17 to 25 °C) and compared to controls held at 21 °C. Fish were exposed to these treatments for 8 or 24 d, and growth and feeding rate, and endocrine and cellular stress responses were examined. To understand the impact of daily temperature oscillations on growth, individuals experienced these treatments for 8 and 24 d. A

second experiment was conducted to examine acute responses and recovery from daily temperature variations. Brook trout were exposed to the same daily temperature variations as above for 1 or 4 d, and then sampled 1 hr after the peak of temperature exposure. in order to understand the effect of acutely elevated temperatures on the stress response. Additional fish were returned to 21 °C and sampled 2, 4, and 10 d later to determine the recovery period following daily temperature oscillations.

Materials and methods

Fish stock

Juvenile (0+) brook trout were obtained from the Sandwich State Hatchery (Sandwich, MA, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in July 2010 and 2011. Fish were housed in 1.7 m diameter tanks supplied with 4 l min^{-1} chilled Connecticut river water (16±2 °C) and given supplemental aeration. Fish were fed to satiation (Zeigler Bros, Gardners, PA, USA) with automatic feeders and maintained under natural photoperiod.

Experiment I: Growth Study

In October 2010 ninety fish were moved from their rearing tank to one of nine 0.6 m diameter experimental tanks (n=10 per tank) and allowed to acclimate for one week prior to the start of the experiment. The fish were fed to satiation once daily and the tanks were supplied with 16 °C Turners Falls, MA city water at a rate of 0.8 l min^{-1}. The water temperature was increased from 16 to 21 °C over 48 hr and then held at 21 °C for 5 d. Each tank received additional heated (34 °C) city water as needed to achieve the desired temperature. The heated water flowed through solenoid valves (Granzow, Inc Charlotte, NC, USA) that were controlled by Omega cn7500 controllers (Omega Engineering, Inc Stamford, CT, USA) with resistance

thermometer input installed on each tank. The controllers where optimized to the testing conditions and programmed to pulse the solenoid valves open and shut at varying frequency to either maintain a set point or to achieve a new set point within a predetermined time frame. Each tank was provided with supplemental aeration.

Feed was withheld from the fish for 24 h prior to the start of the experiment. On the first day of the experiment all of the fish were lightly anesthetized (50 mg MS-222 l^{-1}, pH 7.0) so that length and weight could be recorded. Additionally, each fish received a unique paint mark on either the anal or caudal fin for individual identification purposes. Fish were allowed to recover before being returned to their experimental tanks. The temperature regimes chosen for this study were based on temperature records from known brook trout streams in western Massachusetts (Chadwick, Nislow, and McCormick, unpublished data). The three temperature treatments (3 tanks per treatment) were initiated that evening. Treatment 1 was a control group and was held at 21 °C throughout the study. Treatment 2 consisted of a daily 4 °C oscillation that fluctuated between 19 °C and 23 °C. Treatment 3 consisted of a daily 8 °C oscillation that fluctuated between 17 °C and 25 °C. Treatments 2 and 3 were designed such that the daily low temperature occurred at 0600 and the daily high at 1800 so that the daily average was 21 °C. These temperature treatments were repeated daily for 24 d. Water temperatures were measured and recorded every 15 min using Hobo pendent temperature loggers. Fish were fed to satiation once daily and the amount of feed offered was measured by weight (nearest 0.1 g). Feeding occurred between 1100 and 1200 when all treatments were at 21 °C. Dissolved oxygen levels were measured daily and were always above 90% saturation. On day 6 a valve remained open in one of our three control tanks and the subsequent temperature spike killed all of the fish in the tank.

Every 8 d the fish were measured for length and weight as described. On day 8 four fish per tank were sacrificed using a lethal dose of anesthetic (100 mg MS-222 l^{-1}, pH 7.0) so that blood, plasma and gill, tissues could be sampled as described. In addition to these tissues, the liver was removed, weighed (nearest 0.0001 g), and stored at -80 °C. The remainder of the fish (n=6 per tank) were sampled after 24 d. All sampling occurred between 0900 and 1100.

Experiment II: Acute Exposure

In September 2011 one hundred sixty fish were moved from their rearing tank to one of 10 0.6 m diameter experimental tanks (n=16 per tank) and allowed to acclimate for 11 d prior to the start of the experiment. The fish were fed to satiation once daily and the tanks were supplied with 18 °C Turners Falls, MA city water at a rate of 0.8 l min^{-1}. The water temperature was increased from 18 to 21 °C over 4 d and then held at 21 °C until the start of the experiment. The water temperature in each tank was regulated as described above. Each tank was provided with supplemental aeration.

Feed was withheld from the fish for 24 h prior to the start of the experiment. There were five temperature treatments (2 tanks per treatment) used in this study. The same treatments were used in this study as described above. Treatment 1 was a control group that was maintained at 21 °C throughout the study. Treatment 2 consisted of a daily 4 °C oscillation that fluctuated between 19 °C and 23 °C. Treatment 3 consisted of a daily 8 °C oscillation that fluctuated between 17 °C and 25 °C. Treatments 2 and 3 were repeated daily for 4 d. Treatments 4 and 5 featured the same temperature oscillations as treatments 2 and 3, but these fish only experienced this fluctuation on 1 d and were otherwise kept at 21 °C. On the last day of exposure each treatment reached its peak temperature and was allowed to return to 21 °C where they were held for the remainder of the study in order to investigate recovery time from stressfully elevated temperatures. Temperature treatments were initiated in the morning, so

that peak temperature was reached in the afternoon as would occur in nature. Water temperatures were measured and recorded every 10 min using Hobo pendent temperature loggers (Onset Computer Corporation, Bourne, MA, USA). Dissolved oxygen levels were measured daily in each tank at the peak temperature and were always above 90% saturation.

Four fish per tank (n=8 per treatment) were sampled 1 hr after reaching peak temperature on the 4th day of treatment respectively. Fish were sacrificed using a lethal dose of tricaine methanesulfonate (100 mg MS-222 l^{-1}, pH 7.0) so that tissues could be sampled. Length (nearest 0.1 cm) and weight (nearest 0.1 g) were recorded for each fish. Blood was collected from the caudal vessels using 1 ml ammonium heperanized syringes within five minutes of tank disturbance. The blood was spun at 3200 g for 5 min at 4 °C and the plasma was aliquoted and stored at -80 °C. A biopsy of four to six gill filaments was taken from the first arch and immersed in 100 µl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and stored at -80 °C. Four fish per tank were sampled 2, 4, and 10 d after initial sampling as described. All fish were fed to satiation on the morning after initial sampling (1 d) and at 3, 5, 7, and 9 d and the amount of feed offered was measured by weight (nearest 0.1 g).

Western Blot

Gill Hsp-70 protein abundance was measured as previously described (Pelis and McCormick 2001) using an antibody that is sensitive to the inducible isoform of salmonid Hsp-70 (Rendell et al., 2006). Gill biopsies were homogenized in 150 µl SEID (SEI buffer and 0.1% deoxycholic acid). After grinding, the samples were spun at 5,000 g for 5 min at 4 °C. A small volume of supernatant was used to determine total protein concentration using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). The remaining homogenate was diluted with an equal volume of 2x Laemmli buffer, heated for 15 minutes at 60 °C and stored at -80 °C. Thawed samples were run on a 7.5% SDS-PAGE gel at 2.5 µg total protein per lane with 5 µg

Precision Plus protein standards in a reference lane (Bio-Rad Laboratories, Hercules, CA, USA). For each gel an additional reference sample for Hsp-70 was run. Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membranes (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mM Tris, 192 mM glycine buffer at pH 8.3. PVDF membranes were blocked in phosphate-buffered saline with 0.05% Triton X-100 (PBST) and 5% non-fat dry milk for 1 h at room temperature, rinsed in PBST, and probed with an Hsp-70 antibody (AS05061; Agrisera, Sweden) diluted 1:20,000 in PBST and 5% nonfat dry milk for 1 h at room temperature. This antibody is specific to the inducible isoform of salmonid Hsp-70 and does not recognize the constitutive isoform (Rendell et al., 2006). After rinsing in PBST, blots were exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:10,000 in PBST and 5% nonfat dry milk for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent solution A (ECL A; 396 µM coumaric acid, 2.5 mM luminol, 100 M Tris-Cl pH 8.5) and ECL B (0.018% H_2O_2, 100 mM Tris-Cl pH 8.5), then exposed to X-ray film (RPI, Mount Prospect, IL, USA). Digital photographs were taken of individual gels and band staining intensity measured using ImageJ (NIH, Bethesda, MD, USA); protein abundance is expressed as a cumulative 8-bit gray scale value. The Hsp-70 reference sample run on each gel was used to correct for inter-blot differences.

Plasma Analysis

Plasma glucose was measured by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase (Carey and McCormick 1998). Plasma cortisol was measured by enzyme immunoassay (EIA) as previously described (Carey and McCormick 1998).

Statistics

All data are presented as mean±standard error. For all analyses the probability of establishing statistical significance was $p < 0.05$. All statistical analyses were performed using

Statistica 6.0 (Statsoft, Inc., Tulsa, OK, USA). Daily growth rate in weight was calculated as 100(((natural log of end weight)-(natural log of start weight))/number of days). Daily growth rate in length was calculated as ((end fork length)-(start fork length))/number of days). Hepatosomatic index was calculated as 100(liver weight/body weight). The data from both experiments was analyzed using a 2-way ANOVA with temperature treatment and treatment length as the predictor variables. Where significance was detected a Tukey's HSD post hoc test was run to determine differences among individual groups. Growth rate was analyzed using only data from individuals sampled on day 24. Here a 1-way ANOVA was run using temperature treatment as the predicting variable.

Results

Experiment I: Growth Study

On the first day of the growth study our heating system malfunctioned and peak temperatures were not achieved; however, this was the only time when there was a significant deviation from our target temperatures (Fig. 3.1a, b). There were two mortalities during the experiment. One occurred on day two in one of our control tanks. The other mortality occurred on day eight in one of the 8 °C oscillation tanks.

In individuals sampled at 24 d, growth rate ($mm \bullet d^{-1}$) declined with severity of temperature treatment (Fig. 3.2a; $p = 0.03$). Growth declined with magnitude of temperature oscillation and was 23% and 43% lower at 4 °C and 8 °C oscillation respectively, than in the 21 °C control. We observed a similar trend with regard to specific growth rate. Specific growth rate was 10% and 35% lower in the 4 °C (1.76 $g \bullet d^{-1}$) and 8 °C (1.28 $g \bullet d^{-1}$) oscillations than in the 21 °C (1.95 $g \bullet d^{-1}$) control, but was not statistically significant ($p = 0.07$). Hepatosomatic index increased over the duration of the study, but there were no differences between any of the

treatment groups (Fig. 3.2b; temperature: $p = 0.34$, length: $p < 0.01$). Conversion efficiency was lowest at 8 °C oscillation, where it was 16% lower than in the 21 °C control (Table 3.1).

There was no effect of temperature treatment on plasma cortisol levels (Fig. 3.3a; temperature: $p = 0.78$); however, there was an effect of treatment duration (duration: $p = 0.04$). For all treatments, plasma cortisol was higher at 8 d than it was at 24 d. There was no effect of treatment temperature or duration on plasma glucose (Fig. 3.3b; temperature: $p = 0.37$, duration: $p = 0.06$). Gill Hsp-70 increased with magnitude of temperature oscillation (temperature: $p < 0.01$, duration: $p = 0.10$). Gill Hsp-70 abundance was 40 and 700 fold greater at 4 and 8 °C oscillation than in the 21 °C control.

Experiment II: Acute Exposure

We were close to achieving our target temperatures and there was minimal deviation from the planned daily temperature fluctuations (Fig. 3.1c). There was no effect of temperature treatment on plasma cortisol (Fig. 3.4a; temperature: $p = 0.24$, duration: $p = 0.19$) or plasma glucose (Fig. 3.4b; temperature: $p = 0.25$). Plasma glucose levels did increase over the course of the study (Fig. 3.4b; duration: $p < 0.01$), but this occurred in all temperature treatments and is likely unrelated to the stress response. There was a significant effect of temperature treatment and duration on gill Hsp-70 abundance (Fig. 3.4c)(temperature: $p < 0.01$, duration: $p < 0.01$, interaction: $p < 0.01$). At 1 h gill Hsp-70 abundance corresponded with magnitude and duration of daily temperature oscillation (control < 4 °C oscillation 1 d < 4 °C oscillation 4 d < 8 °C oscillation 1 d < 8 °C oscillation 4 d). After fish were returned to control conditions, gill Hsp-70 abundance decreased over time to those of the control treated individuals. At 4 d Hsp-70 abundance in individuals exposed to 4 °C oscillation had recovered to control levels, whereas those exposed to 8 °C oscillation remained elevated. At 10 d there were no significant

differences between the treatment groups. There was no effect of temperature on hematocrit (temperature: $p = 0.16$; data not shown).

Discussion

Here we demonstrate decreased growth rate in brook trout exposed to daily temperature oscillations in comparison to constant temperature controls held at the same mean temperature. Growth by length and weight declined 43 and 35% respectively at 8 °C oscillation when compared to controls. We did not observe a relationship between magnitude of daily oscillation and plasma cortisol or glucose. Hsp-70 abundance in the gill was 40 and 700 fold greater at 4 °C and 8 °C daily oscillation than in the controls. Induction of the cellular stress response may result in reduced growth.

Our findings of decreased growth with increased temperature oscillation are similar to those reported in Lahontan cutthroat trout where growth declined with increasing magnitude of daily oscillation around a mean of 18 °C (Meeuwig et al., 2004). In this study mass growth rate was 24% and 52% lower in the 6 °C and 12 °C daily oscillation treatments than in 18 °C controls (Meeuwig et al., 2004). A study in Rainbow trout used several constant temperature treatments each with a corresponding 4 °C daily oscillation treatment around the same mean temperature (Hokanson et al., 1977). Here specific growth rate was similar across most of the constant and oscillating treatments; however at 22 °C specific growth rate in the oscillating treatment (-2.12 $g \bullet d^{-1}$) was substantially lower than in the constant control (3.94 $g \bullet d^{-1}$) (Hokanson et al., 1977). This is in agreement with work in coho and Atlantic salmon that found no difference in growth between oscillating and constant temperature treatments, but that featured relatively low peak temperatures of 17 °C and 20 °C respectively (Shrimpton et al., 2007; Thomas et al., 1986). We fed once daily at noon when all of our treatments were at 21 °C whereas all of the studies above

fed more than once per day. It is possible that the timing of our feeding introduced a bias, but there is a limited literature on the relationship between daily temperature oscillation and feeding behavior. There is evidence that salmonids forage nocturnally under cooler conditions and during the day when at elevated temperature and this is primarily attributed to efficiencies in feeding and the ability to avoid predation rather than an optimal temperature for feeding (Bradford and Higgins 2001; Railsback et al., 2005; Young et al., 1997). All of these studies, as well as our own, featured temperature oscillations that are ecologically relevant and the consistency of these findings give validity to the idea that at temperatures elevated beyond the optimum for growth, daily temperature oscillations result in reduced growth in salmonids.

Here we show induction of the stress response as demonstrated by induction of gill Hsp-70 in both short and long term exposures to daily temperature oscillations. Individuals that experienced an 8 °C oscillation for 1 or 4 d had Hsp-70 levels that were 300 and 400-fold that of 21 °C controls. Similar treatments for 8 or 24 d resulted in 40 and 700-fold increase in Hsp-70 abundance compared to controls held at 21 °C for 4 d. Comparable, though less drastic, trends were seen in individuals exposed to 4 °C daily oscillations with a peak at 23 °C. Not only do elevated temperatures induce an Hsp response as many have shown, but our data suggest a cumulative effect of repeated exposure to a stressfully elevated temperature. We did not see induction of Hsp-70 in our controls which supports extensive work from our lab (Chadwick and McCormick, unpublished data) that indicates induction of Hsp-70 at temperatures above 21 °C. Our finding of induction of the Hsp response by elevated temperature is supported by an extensive literature in a variety of salmonids, including Atlantic salmon (Smith et al., 1999), Chinook salmon (Mesa et al., 2002), rainbow trout (Rendell et al., 2006), and brook trout (Lund et al., 2003). There has been little attention to the effect of daily temperature oscillation on Hsp expression in the literature despite the ecological relevance of such treatments. (Cassinelli and

Moffitt 2010) reported greater Hsp-70 abundance in Redband trout exposed to daily oscillations of 18 to 26 °C compared to those exposed 8 to 16 °C daily oscillations. Although the effect of daily temperature oscillation on Hsp expression has received little attention in lab based studies, a significant relationship between water temperature and Hsp-70 abundance has been observed in wild Atlantic salmon, steelhead trout, and redband trout (Feldhaus et al., 2010; Lund et al., 2002; Werner et al., 2005). We have observed increased gill Hsp-70 abundance in brook trout at field sites at which the mean temperature exceeded 21 °C (Chadwick, Nislow, and McCormick, unpublished data).

There has been relatively little attention to the persistence of the Hsp response in fish allowed to recover from a thermal stress despite its importance in validating the use of Hsps as biomarkers for thermal stress in the wild. To our knowledge we are the first to examine recovery of the Hsp-70 response in brook trout following an acute thermal stress. In individuals that experienced an 8 °C oscillation gill Hsp-70 was elevated above controls after 4 d, but not after 10 d. For individuals exposed to a 4 °C oscillation gill Hsp-70 was elevated after 2 d, but had recovered to control levels by 4 d. In the only other paper we know of to have investigated recovery of the Hsp response in fish, liver Hsp-70 remained elevated above controls after 14 d in chinook salmon (Mesa et al., 2002). The difference between these findings and our own could be due to a variety of factors including different species, tissues, temperature treatments, and assays used. The persistence of Hsps following a temperature stress may protect wild fish from subsequent temperature stressors. The fact that the Hsp response lasts for days suggests that it may be a more effective biomarker for detecting temperature stress in wild populations than more common indicators of stress, such as plasma cortisol and glucose, which have a signal that lasts for hours.

We were surprised by the lack of evidence for an endocrine stress response in the current study. There was no difference in plasma cortisol or glucose levels between our controls and our various temperature treatments. We have seen an increase in plasma glucose following an acute temperature exposure in which brook trout were rapidly heated (8 °C h^{-1}) and then held at their target temperatures for 5 h (Chadwick and McCormick, unpublished data). Additionally, we have also seen elevated plasma cortisol levels in individuals exposed to chronically elevated temperatures (Chadwick and McCormick, unpublished data). Furthermore, we have observed elevated plasma cortisol and glucose levels in wild brook trout sampled at sites with elevated temperatures (Chadwick, Nislow, and McCormick, unpublished data). This is in addition to a literature that has shown elevations in plasma cortisol and glucose in response to elevated temperatures in a number of salmonids, including: chinook salmon, sockeye salmon, and rainbow trout (Meka and McCormick 2005; Quigley and Hinch 2006; Steinhausen et al., 2008). In a separate experiment from their growth study, (Thomas et al., 1986) observed elevated plasma cortisol in individuals exposed to a daily oscillation of 6.5 – 20 °C when compared to more moderate daily oscillations and constant temperature controls. On the other hand, redband trout subjected to 8 °C oscillations of 8 to 16 °C or 18 to 26 °C did not exhibit elevated plasma cortisol levels (Cassinelli and Moffitt 2010). It is also possible that there was an endocrine stress response induced during our growth study and that we missed the signal due to the timing of our sampling. In Atlantic salmon plasma cortisol and glucose have been shown to return to baseline levels within hours following an acute crowding stressor (Carey and McCormick 1998). In our growth study we sampled fish in the morning when water temperatures had been below 21 °C for at least 9 hours, potentially giving them time to recover from any thermal stress experienced during the previous day. However in our acute exposures we sampled after 1 h at peak temperatures and still did not observe elevated plasma cortisol or

glucose suggesting that our treatments were not severe enough to induce an endocrine stress response.

The relationship between temperature and the stress response is important because as the climate warms individuals are likely to face daily temperature oscillations that include a brief, but stressful peak temperature. Experiencing such a stressor on a daily basis may lead to decline in performance in important life history traits such as growth that would eventually lead to population level effects of elevated temperature. The correlation between decreased growth and elevated Hsp-70 abundance in the gill is important as it demonstrates decreased performance in individuals exposed to stressfully elevated temperatures. It has been suggested that the Hsp response is not without cost as the rapid and substantial synthesis of Hsps likely requires a large amount of cellular and organismal nutrient stores and also ties up the transcriptional and translation machinery in the cell (Feder and Hofmann 1999). While this hypothesis has not been tested directly, recent studies have demonstrated an attenuated Hsp response in starved fish, including in salmonids, given a temperature challenge, suggesting that a full Hsp response does require adequate nutrient stores (Deng et al., 2009; Han et al., 2012; Piccinetti et al., 2012; Viant et al., 2003). Consumption of resources by the Hsp response could limit anabolic growth. Although we did not detect an endocrine stress response, elevated cortisol levels have been shown to reduce growth and likely act through several pathways including by altering endocrine control of growth (Kajimura et al., 2003b; Peterson and Small 2005a) and feeding (Gregory and Wood 1999; Pankhurst et al., 2008a; Pankhurst et al., 2008b; Peterson and Small 2005a), as well as by altering metabolism (De Boeck et al., 2001; Lankford et al., 2005; Morgan and Iwama 1996; O'Connor et al., 2011; Vanderboon et al., 1991). It is important to note that elevated temperature may act independently of the stress response to limit growth. Standard metabolism in salmonids, as in other ectotherms, has been shown to

increase with temperature (Brett and Glass 1973; Kelsch and Neill 1990). Here we observed decreased conversion efficiency at 8 °C oscillation compared to the 21 °C control suggesting an increased metabolism at 8 °C oscillation. Increased metabolism at elevated temperatures would increase the cost of maintaining homeostasis and thus decrease the nutritional resources necessary for somatic growth.

There is a well established relationship between temperature and growth in brook trout (Baldwin 1956; Brett 1979b; Dwyer et al., 1983; McCormick et al., 1972; McMahon et al., 2007a), including at elevated temperatures (Chadwick and McCormick, unpublished data). However these studies featured fixed treatments in which temperatures were held constant over weeks or months. Here we demonstrate reduced growth in brook trout exposed to daily temperature oscillations that feature peak temperatures that are brief, but stressful. These findings are important as they demonstrate decreased performance in brook trout under temperature regimes that are ecologically relevant and that are likely to become more frequent given predicted climate scenarios (Intergovernmental Panel on Climate Change 2007). Growth is an important life history trait and there is a well established link between body size and fecundity in salmonids (Dickerson et al., 2002; Thorpe et al., 1984). Daily exposure to elevated temperature could reduce growth and fecundity in individuals thus reducing effective population sizes and potentially threatening populations.

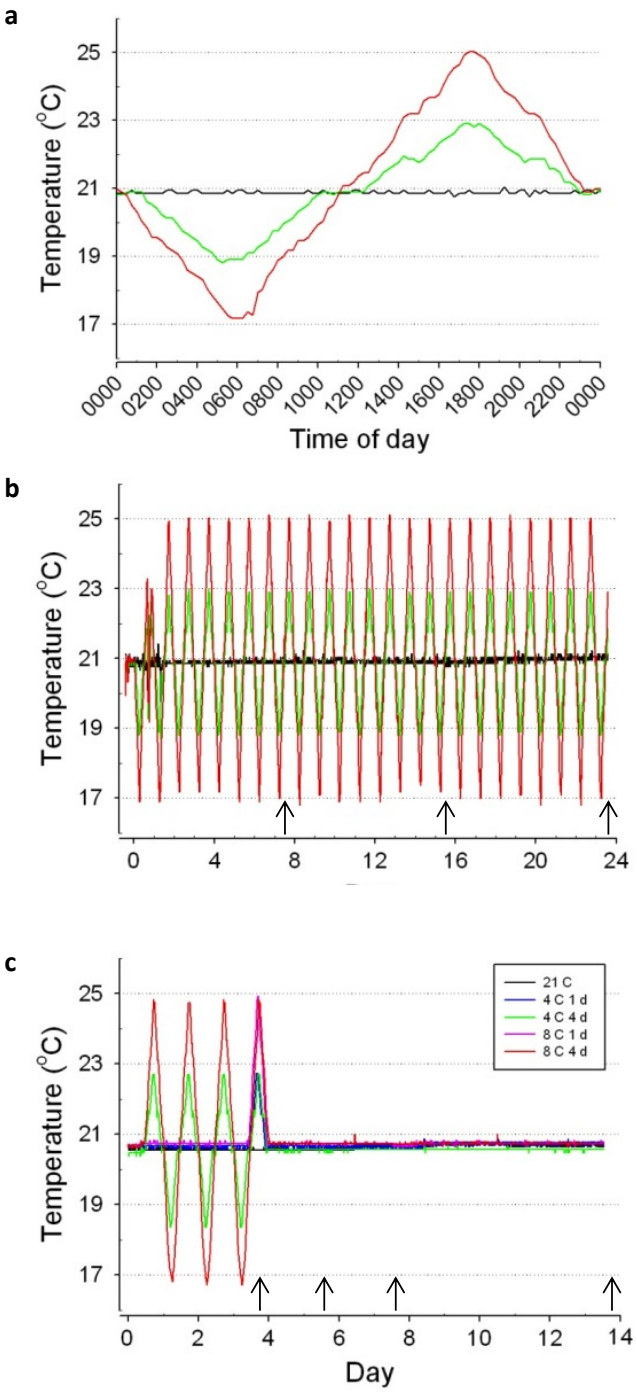

Figure 3.1 | Temperature profiles for the daily temperature treatments. **a**, The temperature profile of a representative day from the growth study. The same profile was used in the acute study. **b**, In the growth study fish were exposed to daily temperature oscillations for 8 or 24 d. **c**, In the acute study brook trout were exposed to daily temperature oscillations for 1 or 4 d. Arrows indicate sampling points.

Table 3.1 | The effect of temperature on feeding and feed conversion efficiency. Feed offered is the mean total dry weight of feed offered per tank for a given treatment over 24 d. Weight gained was the mean total dry weight gained per tank for a given treatment over the 24 d. Conversion efficiency = Weight Gained/Feed Offered.

Temperature Oscillation (°C)	Feed Offered (g)	Weight Gained (g)	Conversion Efficiency
0	104.3	27.1	0.259
4	99.4	27.7	0.279
8	81.4	17.6	0.216

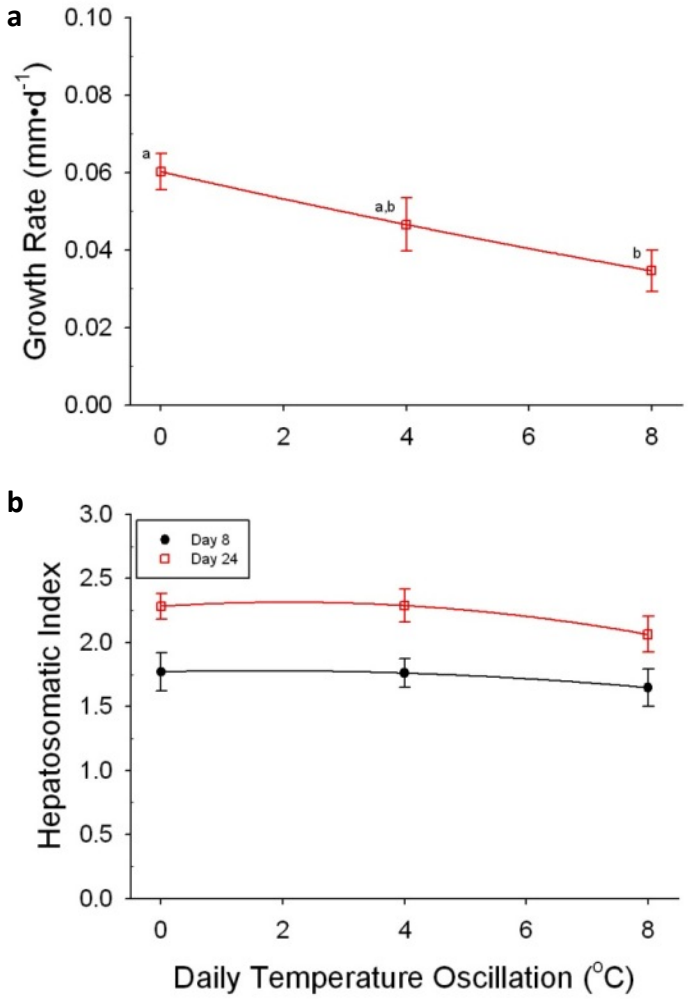

Figure 3.2 | Growth in brook trout exposed to daily temperature oscillations for 8 or 24 d. The effect of daily temperature oscillation for 8 or 24 d on a, growth rate and b, hepatosomatic index. Points represent means±sem (n=8-16). Letters indicate significant differences between temperature treatments.

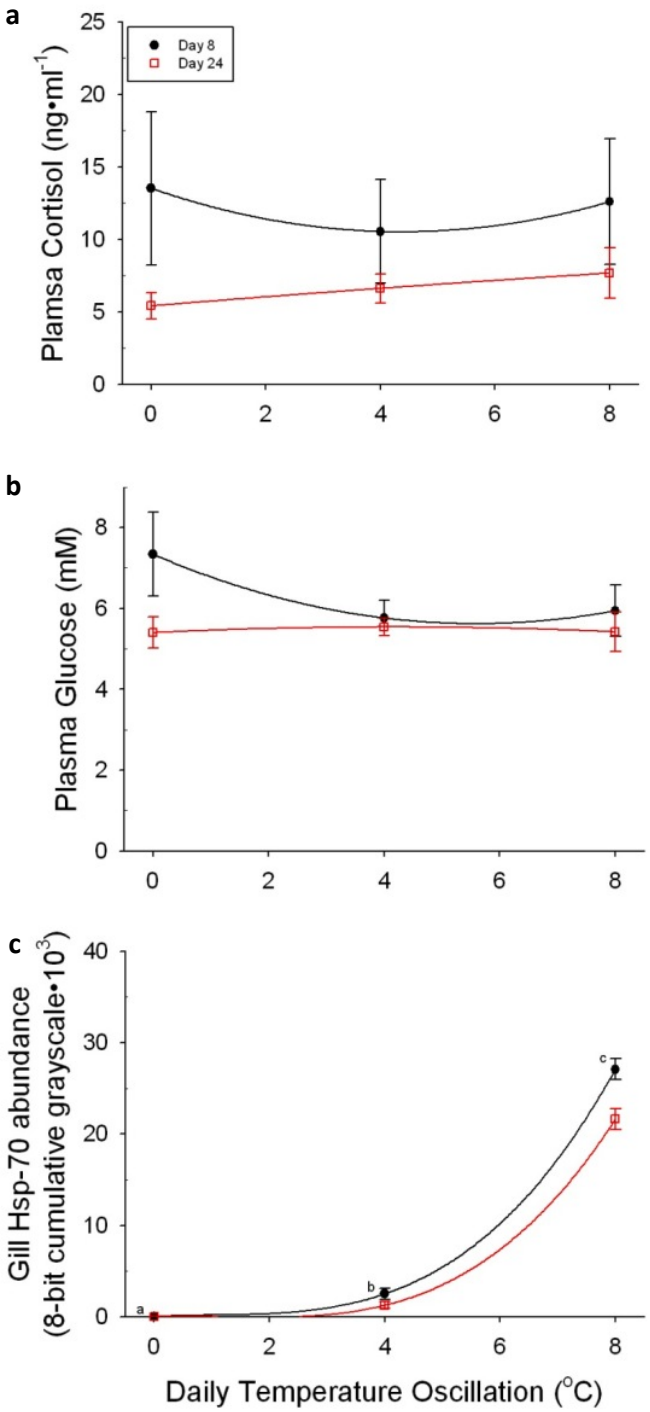

Figure 3.3 | Stress response of brook trout exposed to daily temperature oscillations for 8 or 24 d. The effect of daily temperature oscillation for 8 or 24 d on **a**, plasma cortisol **b**, plasma glucose and **c**, gill Hsp-70 abundance. Points represent means±sem. n=8-16 for plasma cortisol and glucose and n=8 for gill Hsp-70. Letters indicate significant differences between temperature treatments.

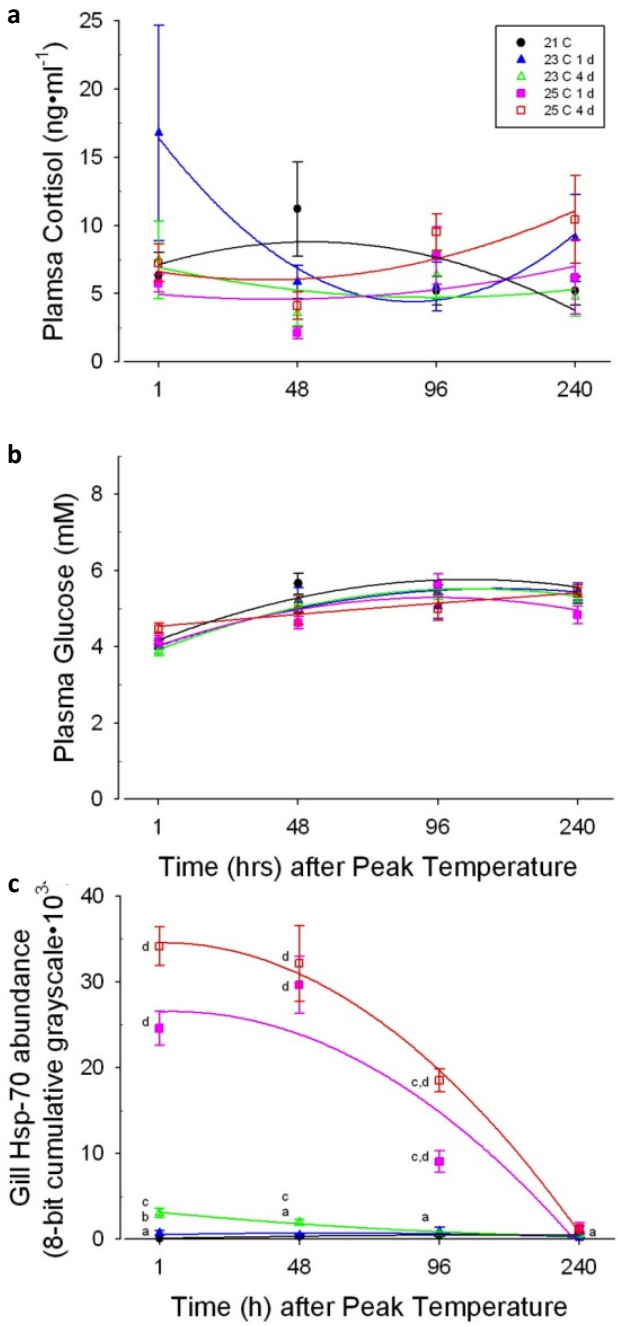

Figure 3.4 | Stress response of brook trout exposed to daily temperature oscillations for 1 or 4 d before being allowed to recover. The effect of daily temperature oscillation for 1 or 4 d on **a**, plasma cortisol **b**, plasma glucose and **c**, gill Hsp-70. Points represent means±sem (n=6-8). Letters indicate significant differences between treatments.

CHAPTER 4

THERMAL ONSET OF THE STRESS RESPONSE CORRESPONDS TO ECOLOGICAL LIMITS IN A COLD-WATER FISH

Climate change is predicted to change the distribution and abundance of species, yet the physiological mechanisms by which environmental temperature drives the distribution of species remain uncertain (Portner and Farrell 2008) and methods for detecting populations at risk from rising temperature are poorly developed. There is increasing interest in using physiological mediators of the stress response as indicators of individual and population level response to environmental stressors (Iwama et al., 1999; Portner and Farrell 2008; Wikelski and Cooke 2006). Here we use laboratory experiments and field assays to show that the temperature thresholds in brook trout (*Salvelinus fontinalis*) for increased gill heat shock protein (Hsp)-70 (20.7 °C) and plasma glucose (21.2 °C) are similar to their proposed thermal ecological limit of 21.0 °C (Wehrly et al., 2007) and that similar temperatures in the wild result in increased plasma glucose, cortisol, and Hsp-70 levels. Furthermore, population densities of brook trout were lowest at field sites where temperatures were warm enough to induce a stress response, and a co-occuring species with a higher thermal tolerance showed no evidence of physiological stress at a warm site. Congruence of stress responses and proposed thermal limits supports the use of these thresholds in models of changes in trout distribution under climate change scenarios and suggests that the induction of the stress response by elevated temperature may play a key role in driving the distribution of species.

Environmental temperature exerts a primary constraint on the distribution and abundance of species. With predicted global increases in temperature, concern has been raised over the future of species whose appropriate thermal habitats will shift and shrink. Indeed, a

number of studies have documented poleward and elevational shifts in species ranges across a variety of taxa (Parmesan and Yohe 2003; Root et al., 2003). Models based on the current relationships between distribution and temperature have been used to estimate the change in species ranges under climate change scenarios. However, uncertainty concerning species thermal thresholds and the presence of multiple limiting factors reduce confidence in these predictions. We contend that there are congruencies between distributional limits and thermal thresholds for the physiological stress response that can substantially increase confidence in such models. Additionally, the stress response can serve as an effective bioindicator of exposure to extreme conditions, which can often be transient, difficult to detect, and uncertain as to their biological effects.

The eastern brook trout may be acutely sensitive to climate change, as it is a cold-water species with populations that are spatially constrained to stream networks. Recent models suggest climate change will lead to a significant loss of brook trout habitat with the greatest reductions occurring in their southern range (Flebbe et al., 2006; Meisner 1990). These models are informed by field observations that indicate that brook trout are rarely found above 21 and 60 day mean temperatures of 22.1 and 21.0 °C (Wehrly et al., 2007). This ecological limit is lower than their upper incipient lethal temperature of 25.3 °C (Fry et al., 1946; Wehrly et al., 2007), suggesting that sub-lethal temperatures play a role in limiting distributions. The physiological mechanisms that underlie this common pattern remain unclear despite the increasing attention to climate change from scientists. Sub-lethal elevated temperatures may act as a stressor, resulting in decreased performance that would lead to population declines.

An extensive literature has identified cortisol as the stress hormone in fish as it is in other vertebrates (Mommsen et al., 1999; Wendelaar Bonga 1997). In response to a real or perceived stressor the hypothalamic-pituitary-interrenal axis is activated and releases cortisol

(Mommsen et al., 1999; Wendelaar Bonga 1997). A limited literature suggests an increase in circulating cortisol and glucose in salmonids in response to elevated temperature (Meka and McCormick 2005; Quigley and Hinch 2006; Steinhausen et al., 2008). In addition to the endocrine stress response there has been increasing attention to the use of aspects of the cellular stress response, such as Hsps, as potential biomarkers for thermal stress (Iwama et al., 1999; Wikelski and Cooke 2006). Inducible isoforms of Hsps are upregulated in the presence of denatured proteins which can result from a variety of environmental stressors including elevated temperature. A number of laboratory studies have identified elevated Hsp expression in response to temperature increases in a variety of salmonid species (Rendell et al., 2006), including in brook trout (Lund et al., 2003). Despite this extensive literature there has been relatively limited exploration of Hsp expression as a biomarker for exposure to elevated temperature in wild fish, though a significant relationship between stream temperature and Hsp-70 has been reported in several salmonids (Feldhaus et al., 2010; Lund et al., 2002; Werner et al., 2005). At present there is limited information on the temperature threshold for the endocrine and cellular stress responses in most fishes and how they relate to their distribution in the wild. In this study, we determined thresholds for stress responses by exposing brook trout to an acute temperature challenge and then tested whether these same responses were manifest in the field by comparing physiological profiles of fish in streams which exceeded temperature thresholds to those in streams which maintained cool temperatures.

In the laboratory, two of the three stress response indicators demonstrated distinct temperature thresholds for induction in brook trout. The temperature threshold for increased gill Hsp-70 was estimated at 20.7 °C, as there was relatively little gill Hsp-70 abundance below 20.7 °C after which Hsp-70 increased rapidly with temperature (Fig. 4.1a, piecewise linear regression, $p < 0.01$, $r^2 = 0.99$) and was greatest at 26 °C. We found no relationship between

temperature and plasma cortisol (Fig. 4.1b, $p = 0.43$), however in a similar study with an 8 day temperature challenge we observed a positive relationship between temperature and plasma cortisol which was elevated at 22 °C. In contrast, plasma glucose increased with temperature (Fig. 4.11c, piecewise linear regression, $p = 0.01$, $r^2 = 0.96$), with a threshold for induction of 21.2 °C.

To demonstrate the efficacy of these biomarkers in wild brook trout and to determine their relationship with overall population density we sampled fish from eight sites within the Connecticut River watershed in Massachusetts in late July 2010 and 2011 (Fig. 4.2a). In general, temperatures were higher in the days preceding the 2011 sampling (Fig. 4.2c) than they were in 2010 (Fig. 4.2b). We observed the highest population density at our coldest site, whereas the three lowest population densities were observed at our three warmest sites (Table 4.1). In 2010 there was relatively little gill Hsp-70 at most sites; however, at the two warmest sites Hsp-70 increased by over two orders of magnitude (Fig. 4.3a, $p < 0.01$, $r^2 = 0.82$). In 2011 Hsp-70 abundance increased with temperature (Fig. 4.3b, $p < 0.01$, $r^2 = 0.30$) and was elevated at the five warmest sites. Interestingly, the lowest seven day mean temperature at which we observed increased Hsp-70 abundance in the field (21.0 °C) was similar to the threshold observed in our lab study. We observed a positive relationship between temperature and plasma cortisol in 2010 (Fig. 4.3c) and 2011 (Fig. 4.3d). In 2011, temperature explained more of the variation in cortisol levels (Fig. 4.3d, $p < 0.01$, $r^2 = 0.19$) than it did in 2010 (Fig. 4.3c, $p = 0.03$, $r^2 = 0.06$), but in both years plasma cortisol increased with temperature. In 2010 we observed an increase in plasma glucose as temperature increased (Fig. 4.3e, $p < 0.01$, $r^2 = 0.17$), but we did not see a significant relationship in 2011 (Fig. 4.3f, $p = 0.45$).

In 2011, we sampled Atlantic salmon (*Salmo salar*) at Roaring Brook 2 and found them to have lower levels for all of our physiological markers for stress (Figs. 4.3b, d, and f) than the

brook trout sampled at the same site. In 2011, we sampled each site a second time in November. Our warmest site was 11.0 °C, whereas the remaining sites were all below 6.0 °C (Fig. 4.S2). All indicators of stress were at or below baseline levels from the summer samples and we did not observe a relationship between temperature and gill Hsp-70 (Fig. 4.S3a, $p = 0.06$), plasma cortisol (Fig. 4.S3b, $p = 0.08$), or plasma glucose (Fig. 4.S3c, $p = 0.65$).

Here we used a laboratory experiment to demonstrate the threshold for the induction of the stress response by elevated temperature in an iconic cold-water fish species. Plasma glucose and gill Hsp-70 increased rapidly at temperatures above 20.7 and 21.2 °C respectively. Additional research from our lab (unpublished results) shows elevated plasma cortisol in brook trout exposed to chronically elevated temperatures. Field sampling conducted at sites representing a range of temperatures also demonstrated a relationship between elevated temperatures and mediators of the stress response. Gill Hsp-70 was greatest in abundance at our warmest sites and was above baseline only at sites where the mean temperature exceeded 21.0 °C. Likewise, plasma cortisol and glucose also exhibited a positive relationship with temperature. This is in contrast to samples collected under cooler conditions where there was no relationship between temperature and any of our indicators of stress. Furthermore, Atlantic salmon sampled at Roaring Brook 2 had lower levels of these indicators of stress than brook trout sampled at the same site. Atlantic salmon are known to have greater thermal tolerances, thus lending further support to our connection between temperature and the stress response in brook trout (Elliott 1991). What is more, our index of population density suggests a relationship between temperature and density where density was greatest are our coolest site and lowest at our warmest sites.

Recent field work suggests that brook trout are rarely seen in streams above 21 and 60 day mean temperatures of 22.1 and 21.0 °C (Wehrly et al., 2007) and in lentic habitats are

limited above 20.0 °C (Robinson et al., 2010), despite the fact that their lethal limit is 25.3 °C (Fry et al., 1946; Wehrly et al., 2007). Here we show that the same temperatures that limit brook trout ecologically also represent the threshold for the stress response in this species. It is likely that brook trout exposed to stressfully elevated temperatures will exhibit altered behavior (Breau et al., 2011; Quigley and Hinch 2006; Wurtsbaugh et al., 1975) and experience decreased performance in important physiological facets such as metabolism (Hartman and Cox 2008), growth (Gregory and Wood 1999; Pickering 1990), and fecundity(Robinson et al., 2010). It is widely accepted that salmonids, including brook trout, exhibit behavioral changes at elevated temperatures as individuals abandon territories to seek out cool water sources, potentially impairing their ability to maximize food resources and avoid predation (Breau et al., 2011; Quigley and Hinch 2006; Wurtsbaugh et al., 1975). It has been suggested that growth in brook trout declines above 16.0 °C (McCormick et al., 1972). Recent work from our lab (unpublished results) strongly supports this idea and shows the upper thermal limit for growth in this species to be 23.4 °C. Growth at upper temperatures is likely to be limited by the stress response, which transfers resources away from somatic growth in order to restore homeostasis (Mommsen et al., 1999; Wendelaar Bonga 1997). The endocrine stress response has also been shown to alter appetite, and may influence endocrine control of growth (Gregory and Wood 1999; Mommsen et al., 1999; Wendelaar Bonga 1997). In fact, it is well established that fish display reduced growth following a variety of stressors (Gregory and Wood 1999; Pickering 1990), and temperature is unlikely to be an exception. There is a well-established relationship between body size and fecundity in salmonids (Thorpe et al., 1984), thus temperature limitations on growth may also limit reproduction. Furthermore, recent field work demonstrates a negative relationship between elevated temperatures and brook trout reproduction(Robinson et al., 2010). These factors represent some of the mechanisms by which sub-lethal yet stressfully

elevated temperatures may act to limit the performance of individuals thus constraining populations and species distributions.

In addition to informing our basic understanding of how species may respond to a warming climate, the knowledge of such temperature thresholds will also help wildlife managers. This knowledge could be used to identify "cool water" sites that may be buffered from the harmful effects of climate change. Likewise, it could help identify populations at risk from rising temperatures. The nonlethal biomarkers validated here could then be used to determine if such populations are in fact experiencing stress from elevated temperatures. These biomarkers could be used in conjunction with or in place of population trend monitoring to justify and evaluate mitigation efforts including the removal of movement barriers and the preservation and restoration of riparian zones in order to stave off the loss of populations due to a warming climate.

Methods

Fifty six juvenile (0+) brook trout were moved from their rearing tank to one of seven 0.6 m diameter experimental tanks (n=8 per tank) and allowed to acclimate at 18 °C for one week. One tank served as a control and was kept at 18 °C. The remaining six tanks were heated at rate of 8 °C h^{-1} until reaching target temperatures of 20, 21, 22, 23, 24, or 26 °C where they were maintained for the remainder of the experiment. The fish were sacrificed 6 h after heating was initiated so that blood and tissue samples could be collected. Nonlethal blood and tissue samples were also collected from wild brook trout sampled in eight streams within the Connecticut River basin in western Massachusetts, U.S.A. in late July 2010 and 2011 and again in November 2011. Gill Hsp-70 abundance was measured by western blotting using an Hsp-70 antibody (AS05061; Agrisera, Sweden) that is specific to the inducible isoform of salmonid Hsp-

70 and does not recognize the constitutive isoform(Rendell et al., 2006). Plasma glucose was measured by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase(Carey and McCormick 1998). Plasma cortisol was measured by enzyme immunoassay (EIA) as previously described(Carey and McCormick 1998). Further details regarding experimental and statistical methods are available as Supplementary Methods.

Supplementary Methods

Fish stock

Juvenile (0+) brook trout were obtained from the Sandwich State Hatchery (Sandwich, MA, USA) and transported to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in July 2011. Fish were housed in 1.7 m diameter tanks supplied with 4 l min^{-1} chilled Connecticut River water (16±2 °C) and given supplemental aeration. Fish were fed to satiation daily (Zeigler Bros, Gardners, PA, USA) with automatic feeders and maintained under natural photoperiod.

Temperature treatment

Fifty six fish were moved from their rearing tank to one of seven 0.6 m diameter experimental tanks (n=8 per tank) and allowed to acclimate for one week prior to the start of the experiment. The fish were fed to satiation once daily and the tanks were supplied with 16 °C Turners Falls, MA city water at a rate of 0.8 l min^{-1}. Each tank received additional heated (~34 °C) city water as needed to achieve the desired target temperature of 18 °C. The heated water flowed through solenoid valves (Granzow, Inc Charlotte, NC, USA) that were controlled by Omega cn7500 controllers (Omega Engineering, Inc Stamford, CT, USA) with resistance thermometer input installed in each tank. The controllers where optimized to the testing conditions and programmed to pulse the solenoid valves open and shut at varying frequency to

either maintain a set point or to achieve a new set point within a predetermined time frame. Each tank was provided with supplemental aeration.

Feed was withheld from the fish for 24 h prior to the start of the experiment. One tank served as a control and was kept at 18 °C throughout the experiment. The remaining six tanks were heated at rate of 8 °C h^{-1} until the target temperatures of 20, 21, 22, 23, 24, or 26 °C were reached (Fig. S1). The water was then held at these target temperatures for the remainder of the experiment. Dissolved oxygen levels were monitored at peak temperature and were found to be above 90% saturation in all tanks.

The fish were sacrificed 6 h after heating was initiated using a lethal dose of anesthetic (100 mg MS-222 l^{-1}, pH 7.0) so that tissue samples could be taken. Fish were measured for length (nearest 0.1 cm) and weight (nearest 0.1 g). Blood was collected from the caudal vessels using 1 ml ammonium heperanized syringes within five minutes of tank disturbance. The blood was spun at 3200 g for 5 min at 4 °C, the plasma was aliquoted and stored at -80 °C. A biopsy of four to six gill filaments was taken from the first arch and immersed in 100 µl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and stored at -80 °C.

Field Sampling

This study was conducted in eight streams within the Connecticut River basin in western Massachusetts, U.S.A. The streams were: Four Mile Brook (second-order, tributary of Connecticut River) in Northfield, MA; Lyons Brook (second-order, tributary of Millers River) Wendell, MA; Collar Brook (second-order, tributary of West Branch Tully River) Royalston, MA; Pond Brook (second-order, tributary of Sawmill River) Montague, MA; Adams Brook (second-order, tributary of Fort River) Amherst, MA; Buffam Brook (first-order, tributary of Fort River) Amherst, MA; Roaring Brook (1) (second-order, tributary of Westfield River) Montgomery, MA; and Roaring Brook (2) (second-order, tributary of Westfield River) Chester, MA. In 2010 these

sites were sampled on July 27-30. The 2011 sampling was done on July 22-26 and again on November 7-10. The temperature and conductivity (YSI Inc) were recorded at each site during sampling. At each site two Hobo pendent temperature loggers (Onset Computer Corporation, Bourne, MA, USA) were placed in the water, one on the upstream and one on the downstream edge of the sampling site and set to record water temperature at forty-five minute intervals.

Sampling was conducted using a one pass electrofishing technique. We only collected samples from individuals that were greater than 8 cm in fork length. This likely meant that during July we were only sampling fish greater than 1 year in age; however, we may have sampled aged 0+ fish during our November sampling. Upon capture, fish were lightly anesthetized with tricaine methanesulfonate (50 mg MS-222 l^{-1}, pH 7.0). Fish were measured for length and weight and bled from the caudal vessel using 1 ml ammonium heperanized syringes within six minutes of capture. The blood was spun at 3200 g for 5 min, the plasma was aliquoted and stored on dry ice. A nonlethal biopsy of four to six gill filaments was taken from the first arch and immersed in 100 µl of ice-cold SEI buffer and frozen on dry ice(McCormick 1993). Fish were allowed to recover before being returned to the stream.

Western Blot

Gill biopsies were homogenized in 150 µl SEID (SEI buffer and 0.1% deoxycholic acid). After grinding, the samples were spun at 5,000 g for 5 min at 4 °C. A small volume of supernatant was used to determine total protein concentration using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). The remaining supernatant was diluted with an equal volume of 2x Laemmli buffer, heated for 15 minutes at 60 °C and stored at -80 °C. Thawed samples were run on a 7.5% SDS-PAGE gel at 2.5 µg per lane with 5 µg Precision Plus protein standards in a reference lane (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membranes (Millipore,

Bedford, MA, USA) at 30 V overnight in 25 mM Tris, 192 mM glycine buffer at pH 8.3. PVDF membranes were blocked in phosphate-buffered saline with 0.05% Triton X-100 (PBST) and 5% non-fat dry milk for 1 h at room temperature, rinsed in PBST, and probed with an Hsp-70 antibody (AS05061; Agrisera, Sweden) diluted 1:20,000 in PBST and 5% nonfat dry milk for 1 h at room temperature. This antibody is specific to the inducible isoform of salmonid Hsp-70 and does not recognize the constitutive isoform(Rendell et al., 2006). After rinsing in PBST, blots were exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:10,000 in PBST and 5% nonfat dry milk for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent solution A (ECL A; 396 µM coumaric acid, 2.5 mM luminol, 100 M Tris-Cl pH 8.5) and ECL B (0.018% H2O2, 100 mM Tris-Cl pH 8.5), then exposed to X-ray film (RPI, Mount Prospect, IL, USA). Digital photographs were taken of films and band staining intensity measured using ImageJ (NIH, Bethesda, MD, USA); protein abundance is expressed as a cumulative 8-bit gray scale value. A reference sample was run on each gel and was used to correct for inter-blot differences.

Plasma Analysis

Plasma glucose was measured by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase(Carey and McCormick 1998). Plasma cortisol was measured by enzyme immunoassay (EIA) as previously described(Carey and McCormick 1998).

Statistics

All data are presented as mean±standard error. Where necessary data was log-transformed and the corresponding p and r^2 values were reported. All statistical analyses were performed using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA) except for the mixed linear model analysis which was performed using R (R Foundation for Statistical Computing, Vienna, Austria). For all analyses the probability of establishing statistical significance was p <

0.05 and when significant effects were observed the r^2 value was reported. For the laboratory portion of this study the data was analyzed using a piece-wise regression with mean temperature serving as a continuous predictor variable.

For the field portion of this study the Hsp-70 data was analyzed using a quadratic regression whereas the cortisol and glucose data were analyzed using a linear regression. Here mean temperature for the 7 d prior to sampling was used as a predictor variable. We also ran the same tests with 2, 3, 10, and 14 d mean temperature as the predictor variable. The explanatory power of the model was greater at 7 d than at 2 or 3 d, but did not show further improvement at 10 or 14 d. Recent work from our lab (unpublished results) shows elevated gill Hsp-70 4 d, but not 10 d after a temperature stressor further supporting the use of the 7 d mean temperature in our field analyses. The July field data from both years was pooled and analyzed using mixed linear model analysis with year, site, and temperature as fixed factors, and weight as a random factor. AIC weights were used to determine that the best model for predicting Hsp70, and cortisol levels included 7 d mean temperature as a predictor variable, thus justifying our use of temperature as a predictor variable in our regression models. For data from the mixed linear analyses see Table 4.1-3s for cortisol, glucose, and Hsp-70.

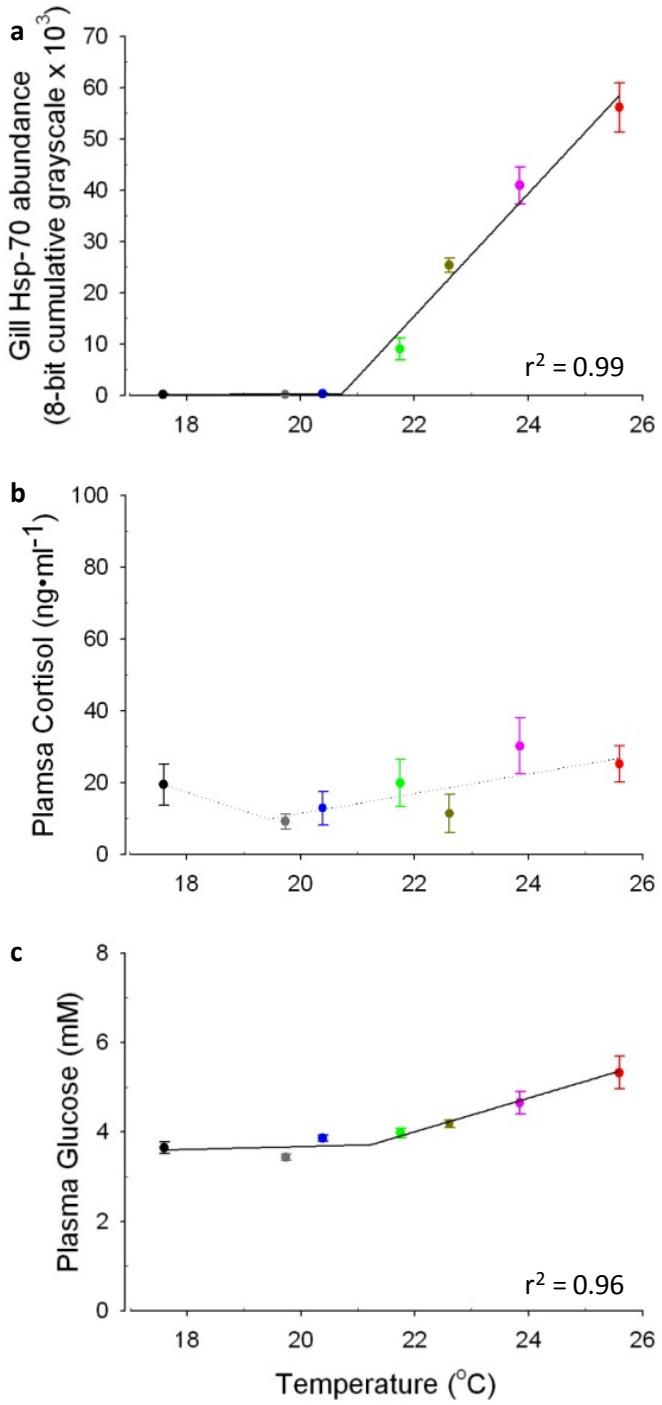

Figure 4.1 | Effect of temperature on the stress response in brook trout. Points represent mean (n=6-8) ± s.e.m. A piecewise regression using temperature as a predictor variable was used to determine temperature thresholds. **a**, The relationship between gill Hsp-70 abundance and temperature showed a threshold for induction of 20.7 °C. **b**, The effect of temperature on plasma cortisol. **c**, The relationship between plasma glucose and temperature showed a threshold for induction of 21.2 °C.

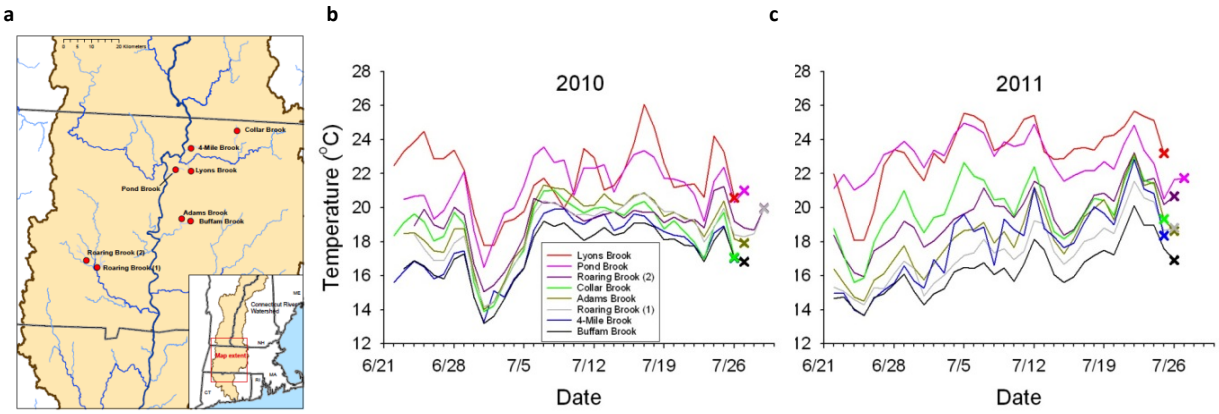

Figure 4.2 | Location and temperature profiles of our 8 field sites. **a**, The location of our 8 field sites. The daily mean water temperature of our field sites in summer **b**, 2010 and **c**, 2011 during the month preceding sampling. The date that each site was sampled is marked by an X.

Table 4.1 | Physical characteristics and population estimates for the 8 field sites as measured during July 2010 and 2011. Temperature is the mean temperature from the week proceeding sampling in 2010 and 2011. Length refers to the length of stream section that we sampled. Here we report width as the mean wetted width of three measurements taken over the length of stream section sampled. We only sampled aged 1+ and older fish. Density was calculated using data from 2010 and 2011.

Site	Temperature (°C)	Length (m)	Width (m)	Density (fish per km^2)
Lyons Brook	23.3	172.0	3.1	13.3
Pond Brook	21.9	336.5	2.9	11.1
Roaring Brook (2)	20.6	175.5	5.5	8.3
Collar Brook	19.8	95.0	3.4	30.2
Adams Brook	20.0	90.5	5.9	18.5
Roaring Brook (1)	19.3	50.0	8.3	24.0
4-Mile Brook	19.3	115.0	5.0	17.4
Buffam Brook	18.0	42.0	3.2	80.6

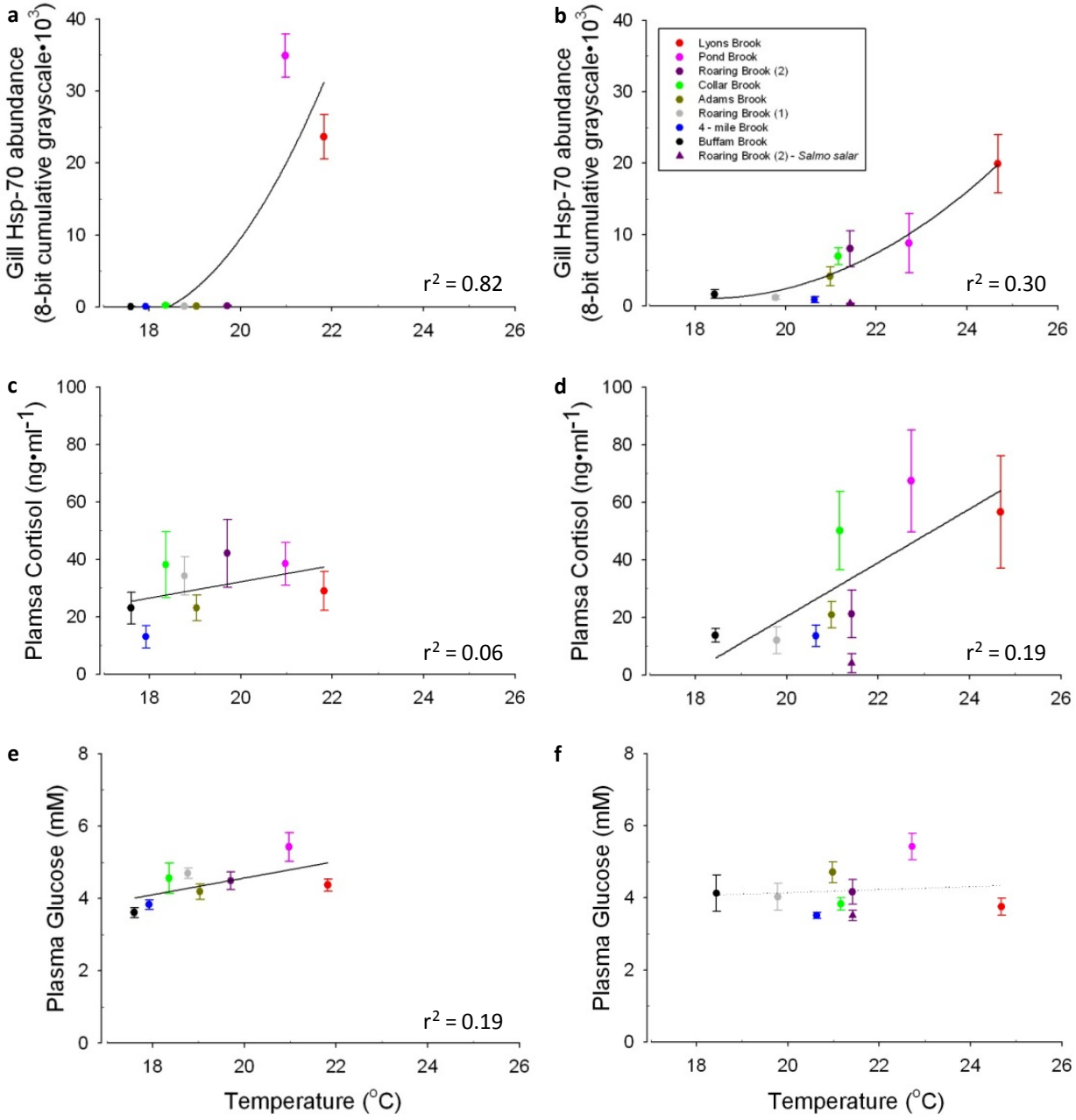

Figure 4.3 | Effect of temperature on the stress response in wild brook trout. Points represent mean (n=6-12) ± s.e.m. Quadratic (**a, b**) and linear regressions (**c-f**) were used to analyze the data. The mean temperature from the seven days preceding sampling was used as a predictor variable. The relationship between gill Hsp-70 and temperature in **a**, 2010 and **b**, 2011. The effect of temperature on plasma cortisol levels in **c**, 2010 and **d**, 2011. The effect of temperature on plasma glucose **e**, 2010, and **f**, 2011.

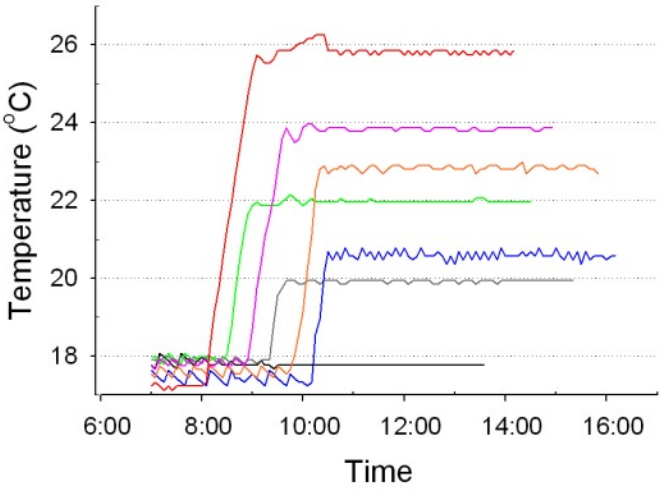

Figure 4.S1 | Temperature treatment of brook trout in the lab. Water temperatures were elevated at a rate of 8 °C h^{-1} until target temperatures were achieved. Fish (n=6-8 per treatment) were sampled 6 h after initiation of heating.

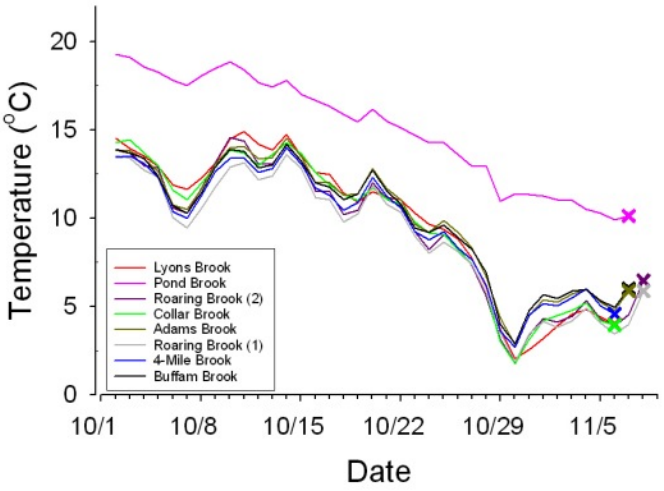

Figure 4.S2 | Temperature profiles of our 8 field sites. The daily mean water temperature at each of our field sites in fall 2011 during the month preceding sampling.

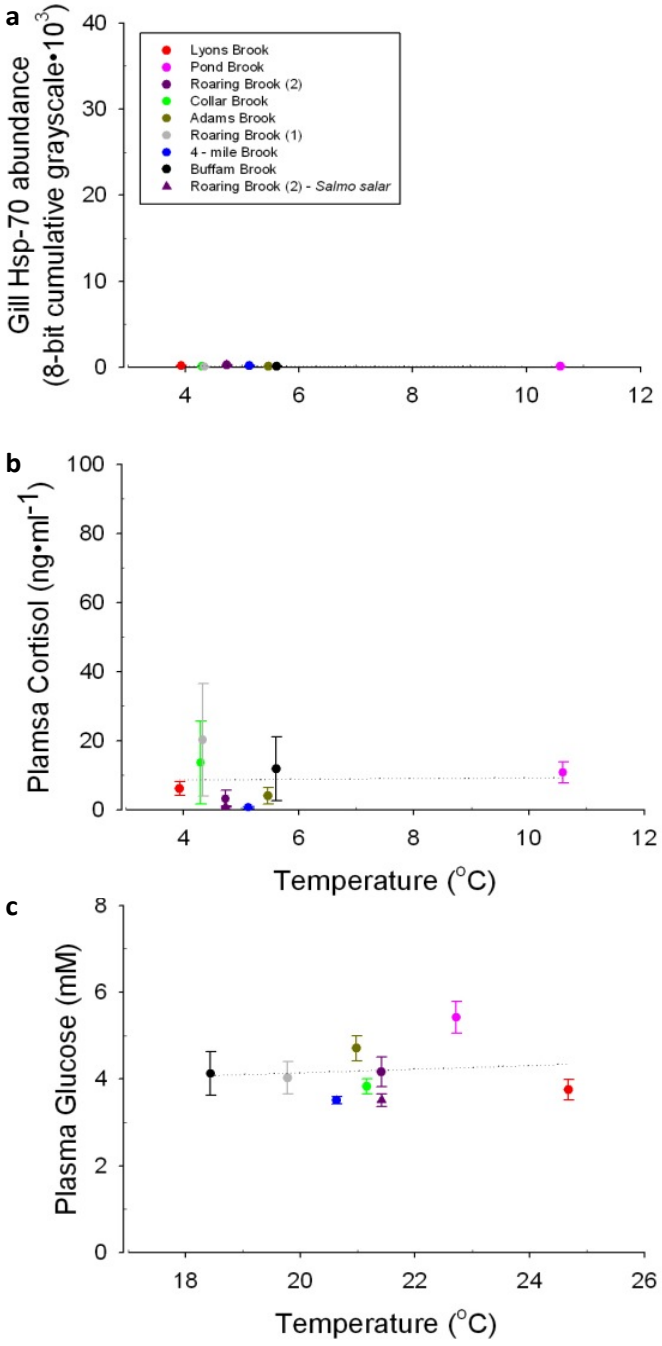

Figure 4.S3 | Effect of temperature on the stress response in wild brook trout in fall 2011. Points represent mean (n=6-10) ± s.e.m. Quadratic (**a**) and linear regressions (**b, c**) were used to analyze the data. The mean temperature from the seven days preceding sampling was used as a predictor variable. The effect of temperature on **a**, gill Hsp-70, **b**, plasma cortisol, and **c**, plasma glucose.

Table 4.S1 | Linear mixed models for plasma cortisol levels in brook trout sampled at 8 sites in western Massachusetts during July 2010 and 2011. Temp is the mean daily temperature for the 7 days preceding sampling. K is the number of parameters in the model, AIC_c is the corrected Akaike's Information Criterion, Δ_i is the difference between model *i* and the most-supported model, ω_i is the Akaike weight of the model. Weight was included as a random term. The most-supported model is marked in bold.

Model	Year	Site	Temp	K	AIC_c	Δ_i	ω_i
1	**X**		**X**	**3**	**136.5**	**0.0**	**0.584**
2				1	138.4	1.9	0.226
3			X	2	139.2	2.7	0.148
4	X			2	142.2	5.7	0.034
5	X	X		3	146.7	10.2	0.004
6	X	X		3	151.1	14.6	0.000
7	X	X	X	4	152.1	15.6	0.000
8		X	X	3	153.4	16.9	0.000

Table 4.S2 | Linear mixed models for plasma glucose levels in brook trout sampled at 8 sites in western Massachusetts during July 2010 and 2011. K is the number of parameters in the model, AIC_c is the corrected Akaike's Information Criterion, Δ_i is the difference between model *i* and the most-supported model, ω_i is the Akaike weight of the model. Weight was included as a random term. The most-supported model is marked in bold.

Model	Year	Site	Temp	K	AIC_c	Δ_i	ω_i
1				**1**	**-275.1**	**0.0**	**0.685**
2	X			2	-268.4	6.7	0.024
3			X	2	-265.6	9.5	0.006
4	X		X	3	-262.8	12.3	0.001
5		X		2	-253.9	21.2	0.000
6	X	X		3	-247.0	28.1	0.000
7		X	X	3	-246.4	28.7	0.000
8	X	X	X	4	-239.7	35.4	0.000

Table 4.S3 | Linear mixed models for gill Hsp-70 levels in brook trout sampled at 8 sites in western Massachusetts during July 2010 and 2011. K is the number of parameters in the model, AIC_c is the corrected Akaike's Information Criterion, Δ_i is the difference between model *i* and the most-supported model, ω_i is the Akaike weight of the model. Weight was included as a random term. The most-supported model is marked in bold.

Model	Year	Site	Temp	K	AIC_c	Δ_i	ω_i
1			**X**	**2**	**271.1**	**0.0**	**0.408**
2	X	X		3	271.6	0.5	0.317
3	X	X	X	4	273.8	2.7	0.106
4	X		X	3	274.1	3.0	0.091
5		X	X	3	274.4	3.3	0.078
6		X		2	325.2	54.1	0.000
7	X			2	364.3	93.2	0.000
8				1	383.6	112.5	0.000

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